A role for CD44 in lymphocyte development and function

By

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Abstract

CD44 is a transmembrane cell surface glycoprotein which has been implicated in various cell processes such as cell adhesion, lymphocyte migration and lymphocyte activation. However, although an important function for CD44 has been suggested by antibody-blocking studies, $CD44^{-/-}$ mice paradoxically show few defects in immune function.

Since compensatory mechanisms, operable in the complete absence of CD44 expression, could account for these apparently contradictory findings, we compared the development and function of $CD44^{+/+}$ and $CD44^{-/-}$ lymphocytes when these cells were in direct competition. Radiation bone marrow chimeras made with different combinations of $CD44^{+/+}$ and $CD44^{-/-}$ donor and recipient mice were employed.

$CD44^{-/-}$ progenitor cells were able to fully reconstitute both T and B cell pools when injected into irradiated $CD44^{+/+}$ or $CD44^{-/-}$ recipients. However, chimeras reconstituted with a mixture of $CD44^{-/-}$ and $CD44^{+/+}$ progenitors showed a deficiency in the generation of $CD44^{-/-}$ T cells. This deficit appeared to be due to both reduced thymic homing and impaired intrathymic maturation of $CD44^{-/-}$ precursors. In contrast, a slight increase in the production of B cells derived from the $CD44^{-/-}$ progenitors was observed in mixed chimeras, which was independent of precursor trapping in the thymus, as shown in experiments in thymectomised mice.

The function of mature $CD44^{-/-}$ lymphocytes was investigated by assessing immune responses after immunisation or infection of mixed chimeras. As measured by tetramer and intracellular cytokine staining, $CD44^{-/-}$ $CD8^{+}$ T cells were shown to respond less well than $CD44^{+/+}$ cells, while $CD44^{-/-}$ B cells exhibited a subtle defect in antibody production.

Overall, the results demonstrate that CD44 plays a role during lymphocyte development and in the function of mature lymphocytes, which is only apparent when CD44 positive and negative cells are in direct competition.

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Abbreviations used

2ME	2-beta-mercaptoethanol
AICD	Activation induced cell death
BM	Bone marrow
BMDCs	Bone marrow derived dendritic cells
CD	Cluster of differentiation
CFSE	5-carboxyfluorescein diacetate succinimidyl ester
CGG	Chicken gamma globulin
CO_2	Carbon dioxide
DC	Dendritic cell
dLN	Draining lymph node
DN	Double negative
DNase	Deoxyribose nucleic acid
DNP	Di-nitro-phenol
DP	Double positive
DTH	Delayed type hyposensitivity
EDTA	Ethylendiaminetetra-acetic acid disodium salt
ELISA	Enzyme linked immunosorbant assay
ELISPOT	Enzyme linked immunosorbant spots
FACS	Fluorescence assisted cytometry sorter
FBS	Foetal bovine serum
Fc	Crystallised fragment
FCS	Foetal calf serum
GM-CSF	Granulocyte macrophage colony stimulating factor

HA	Hyaluronic acid
HSC	Haematopoietic stem cell
I.p	Intraperitoneal
I.t	Intrathymic
I.v.	Intravenus
ICS	Intracellular staining
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LCMV	Lymphocytic choriomeningitis virus
LE	Low endotoxin
LM	Litter mate
LN	Lymph nodes
LPS	Lipopoly saccaride
Ly	Lymphocyte antigen
MFI	Mean fluorescence intensity
mLN	Mesenteric lymph nodes
MLR's	Mixed leukocyte reaction
MZB	Marginal zone B cell
OPD	Optical density
OVA	Ova albumin
PBS	Phosphate buffered saline
PC	Peritoneal cavity
PFA	Paraformaldehyde

pfu	Plaque forming units
pLN	Peripheral lymph nodes
Poly I:C	Poly inosine poly cytosine
RAG	Recombination activation gene
RPMI	Rosemount park memorial institute
s.e.m.	Standard error of the mean
s.d.m	Standard deviation of the mean
SF	Spot forming
sIgD	Surface immunoglobulin D expression
sIgM	Surface immunoglobulin M expression
SP	Single positive
TCR	T cell receptor
VSV	Vesticular stromal virus

Nucleic Acid Symbols

A	Adenosine
T	Tyrosine
U	Uridine
G	Guansine
C	Cytosine

Amino Acid Symbols and Molecular Weight

Amino Acid	3-letter code	1-letter code	Molecular weight (g/mol)
Alanine	Ala	A	89.1
Arginine	Arg	R	174.2
Asparagine	Asn	N	132.1
Aspartate	Asp	D	133.1
Cysteine	Cys	C	121.2
Glutamate	Glu	E	147.1
Glutamine	Gln	Q	146.2
Glycine	Gly	G	75.1
Histidine	His	H	155.2
Isoleucine	Ile	I	131.2
Leucine	Leu	L	131.2
Lysine	Lys	K	146.2
Methionine	Met	M	149.2
Phenylalanine	Phe	F	165.2
Proline	Pro	P	115.1
Serine	Ser	S	105.1
Threonine	Thr	T	119.1
Tryptophan	Trp	W	204.2
Tyrosine	Tyr	Y	181.2
Valine	Val	V	117.1

1. Introduction

1.1.Organisation and development of the immune system

1.1.1. An overview of the immune system.

The immune system has over time evolved to assist the body's defence against harmful invading pathogens, and as the body has become more complex, so has the immune system. The body must therefore have a system in place that can provide protection against a multitude of pathogens without damaging itself. When this regulation is disrupted, damage to the body is caused by the immune system attacking self and causing autoimmune diseases or by "overreacting" to harmless antigens, which results in allergy.

In higher organisms such as mammals, the immune system can be divided into two parts, the innate and the adaptive. The innate immune system is the first to respond to pathogens, mainly microbes. It is the most primitive part of the immune system and comprises mainly of phagocytic cells such cells as macrophages. These cells phagocytose the invading microbes and produce anti-microbial factors such as cytokines, which alert neighbouring cells of potential attack. Certain cytokines also recruit other innate and adaptive cells to the area of infection. After physical barriers such as epithelia and mucosal secretions, the innate immune system is the first line of defence against invading pathogens. Mediators of innate immunity recognise conserved molecules or motifs on microbes that identifies them as pathogenic allowing for an immediate response. This response may be

sufficient to prevent the initial colonisation of the body by the invading microbes, and in instances when the innate immune system is not successful in containing the pathogens, allows time for an adaptive immune response to be mounted. Although innate immunity functions rapidly, there is no recall or 'memory' of the microbes encountered. In addition, since innate pathogen receptors are germ-line encoded, recognition is restricted to pathogen structures that cannot be altered without loss of viability, and so do not adapt to microbial variation.

Conversely, the adaptive immune response can adapt to recognise pathogen variation. Receptors on lymphocytes of the adaptive immune system are generated through somatic recombination of multiple gene segments so that they are able to recognise an almost limitless variety of antigenic molecules, or antigens. A drawback to having such a wide variety of cells is that there are only low numbers of lymphocyte specific for any particular antigen. For this reason, there is a requirement for antigen specific cells to undergo multiple divisions following recognition of antigen in order to generate a sufficient population of pathogen reactive cells. Therefore the adaptive immune response is much slower than the innate response, at least on first encounter with a pathogen. On the other hand, antigen specific lymphocytes persist at a high frequency as memory cells after pathogenic clearance, and provide a more rapid response to re-infection.

There are two parts of the adaptive immune system, the humoral and the cellular arms. The humoral system is comprised of antibodies, secreted into the blood system and mucosal secretions by B cells, which can block and disable invading pathogens and toxins. The cellular arm is mediated by T cells, which recognise infected cells. Effector T cells travel to the site of infection and release chemical

factors which can kill the target cell. At the start of an infection, both aspects of the immune response are typically initiated in secondary lymphoid organs, such as lymph nodes (LN), where fragments of pathogen components are presented to naïve T cells by specialised antigen-presenting cells (APCs), mainly dendritic cells (DCs). Antigen recognition by naïve cells leads to activation and cell multiplication. The resultant population of T cells travels to the site of infection where it interacts with infected cells and destroys the recognised pathogen. Responding B cells secrete immunoglobulins (which bind to the pathogen and label it for phagocytosis or complement cytotoxicity).

1.1.2. Development of haematopoietic stem cells

The cells of both the adaptive and innate immune system are derived from self-renewing haematopoietic stem cells (HSC). Early In embryo formation, three germ layers are formed, the ectoderm, the endoderm and the mesoderm (1). HSC are derived from the mesodermic layer and are capable of generating all erythroid, myeloid and lymphoid cell lineages. As these cells are undifferentiated, they do not express lineage specific cell markers on the cell surface, such as CD3 or CD19; in practical terms this enables cellular enrichment by negative selection. HSCs are thought to be non-cycling and are unresponsive to a multitude of growth factors such as IL-1, IL-3, IL-4, IL-6, CSF-1, G-CSF, and GM-CSF (2). The differentiation of HSC to specific lineages involves multiple transcription factors (Figure 1.1) (3). For example, the transcription factor PU-1 is necessary for differentiation of the HSC's into myeloid lymphoid progenitors (4), while high levels

of Pu-1 and BLIMP-1 in the myeloid lymphoid progenitor are implicated in the differentiation into a myeloid progenitor. These myeloid progenitors can develop into myeloid cells such as macrophages (5, 6), and with the aid of other transcription factors such as Notch3, into mast cells (7). Low levels of Pu-1 and high levels of the transcription factor ikaros are thought to differentiate the lymphoid myeloid progenitor into a common lymphoid progenitor (8, 9). One way that Pu-1 is thought to help differentiate the lymphoid myeloid progenitor is by altering the cells responsiveness to IL-7, since low levels of PU-1 present in the myeloid lymphoid progenitor activates IL-7Rα gene, whereas high levels of PU-1 prevents IL-7Rα expression (10, 11). The Ikaros transcription factor is involved in regulating genes essential in T and B cell development, including gene rearrangement genes such as RAG and TdT, essential B cell genes e.g. Ig-H and Ig-L, as well as T cell CD3 complex genes. It is from this common lymphoid progenitor in normal development that the T and B cell lineages diverge. The existence of a common lymphoid progenitor is not a matter of consensus; this debate is reviewed by Katursa (12).

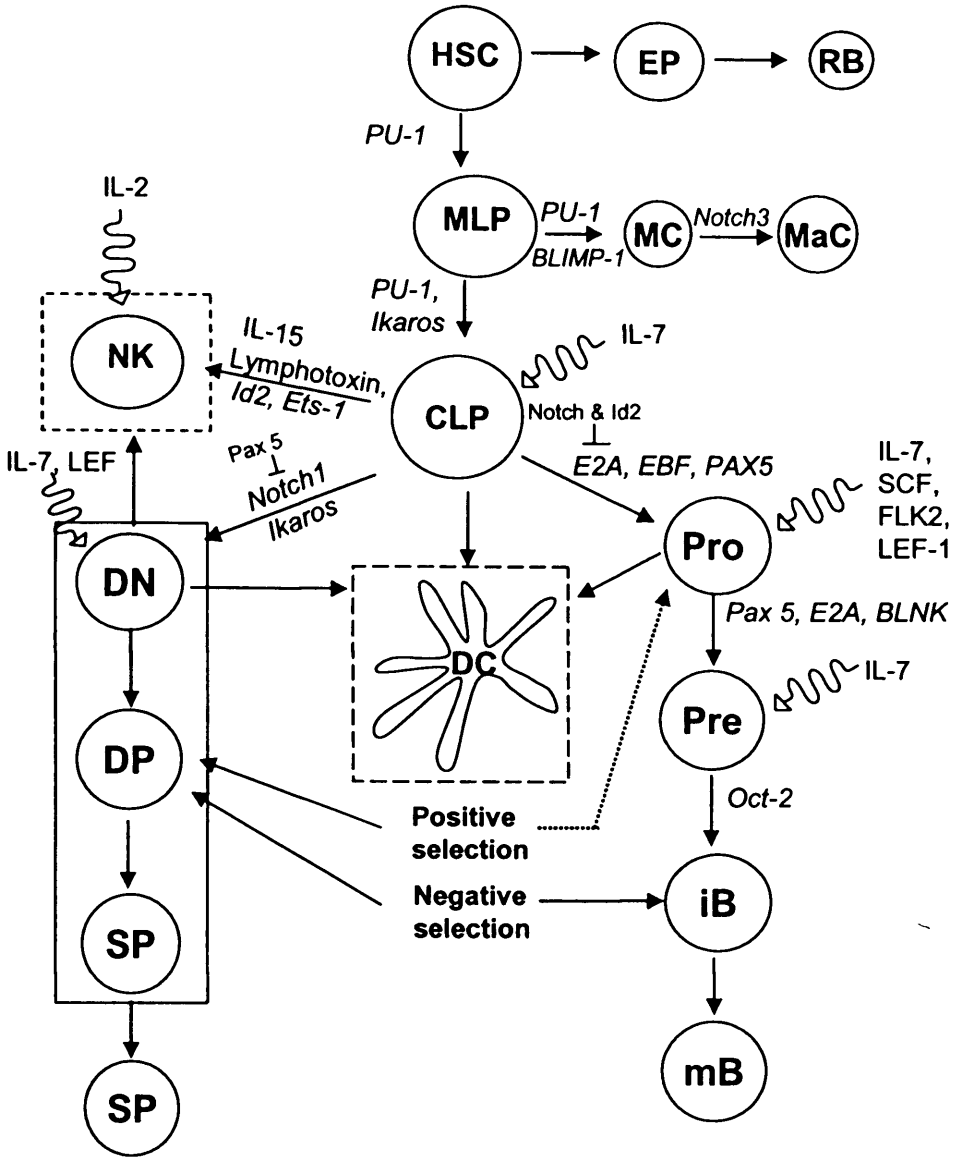

Figure 1.1. Diagram of transcription factors and cytokines involved in haematopoiesis and B and T cell development.

EP, erythroid progenitor. RB, red blood cell. MLP, myeloid lymphoid progenitor. CLP, common lymphoid progenitor. MC, myeloid cells. Mac, Mast cells. NK, natural killer cells. DN, Double negative T cell. DP, double positive T cell. SP single positive T cell. Pro, pro-B cell. Pre, pre-B cell. iB, immature B cell. mB, Mature recirculating B cell. T cells enclosed in box represent thymic development. DC and NK enclosed in a broken box indicates bone marrow or thymic development. ⋯▸ indicates potential positive selection of B-1a cells. ⟶ indicates differentiation. ⟿ indicate action of cytokines. ⊥ Indicates a blocking action

1.1.3. Development of B cells

There are two types of B cells: the B-1 and B-2 B cells. The majority of B cells are B-2 and are the cells typically involved in T-dependent immune responses. The B-1 cells are considered more primitive and are self-renewing. They are not thought to derive from HSC cells. B-1 cells are mainly distributed in the mucosal tissues and the peritoneal cavity. B-2 cells are discussed first.

1.1.3.1. B2 B cells

The differentiation from the common lymphoid progenitor to the first stage of B cell development is dependent on the transcription factors E2A, EBF and Pax 5 (3). It is thought that E2A and EBF act by activating important B cell genes, such as RAG1 and RAG2 which co-ordinate with E2A and EBF to control V(D)J recombination. Pax 5 encourages B cell development by blocking Notch1 gene expression and therefore T cell development (13, 14). Notch1 is not the only lineage-inducing gene that Pax 5 inhibits. Pax 5 also blocks expression of M-CSF-R (monocyte-colony stimulating factor receptor), which therefore inhibits the pro-B cells from developing into monocytes (15).

Mature B cells express immunoglobulins at the cell surface which serve as antigen receptors. Immunoglobulins are made up of two heavy chains and two light chains. There are two types of light chain, κ and λ. The light chain consists of variable (V), joining (J), and constant (C) regions. V and J regions consist of multiple gene segments that undergo somatic recombination, during B cell development, to create a functional light chain. This excision and rejoining of sections generates diversity of the immunoglobulin. The heavy chain consists of V,

diversity (D), J and C regions. The V, D and J regions of the heavy chain also consists of multiple gene segments which undergo somatic recombination and rejoining to generate a diverse functional chain.

The pro-B cell stage is the earliest stage of B cell development, and according to Hardy, can be divided into three stages (Figure 1.2). At the second pro-B cell stage, the heavy chain locus undergoes D-J rearrangement to form a V-DJ heavy chain. At the third pro-B cell stage V-DJ heavy chain rearrangement occurs to form a VDJ rearrangement. During rearrangement, double stranded breaks in the DNA are initiated by RAG1 and RAG2 proteins at a specific sequence, a hairpin structure is formed that is 'nicked' open and subjected to varying amounts of digestion, before TdT adds a random number of nucleotides to the 3' DNA strands. The DNA polymerase fills in the partner strand before the two ends are ligated together. The processes digestion and TdT activity add more diversity to the resulting of immunoglobulins. Successful heavy chain gene rearrangement allows the cell to receive survival and proliferative signals upon expression if the receptor on the cell surface, while unsuccessful rearrangements (for example, when the heavy chain is out of frame) force the cell to undergo apoptosis.

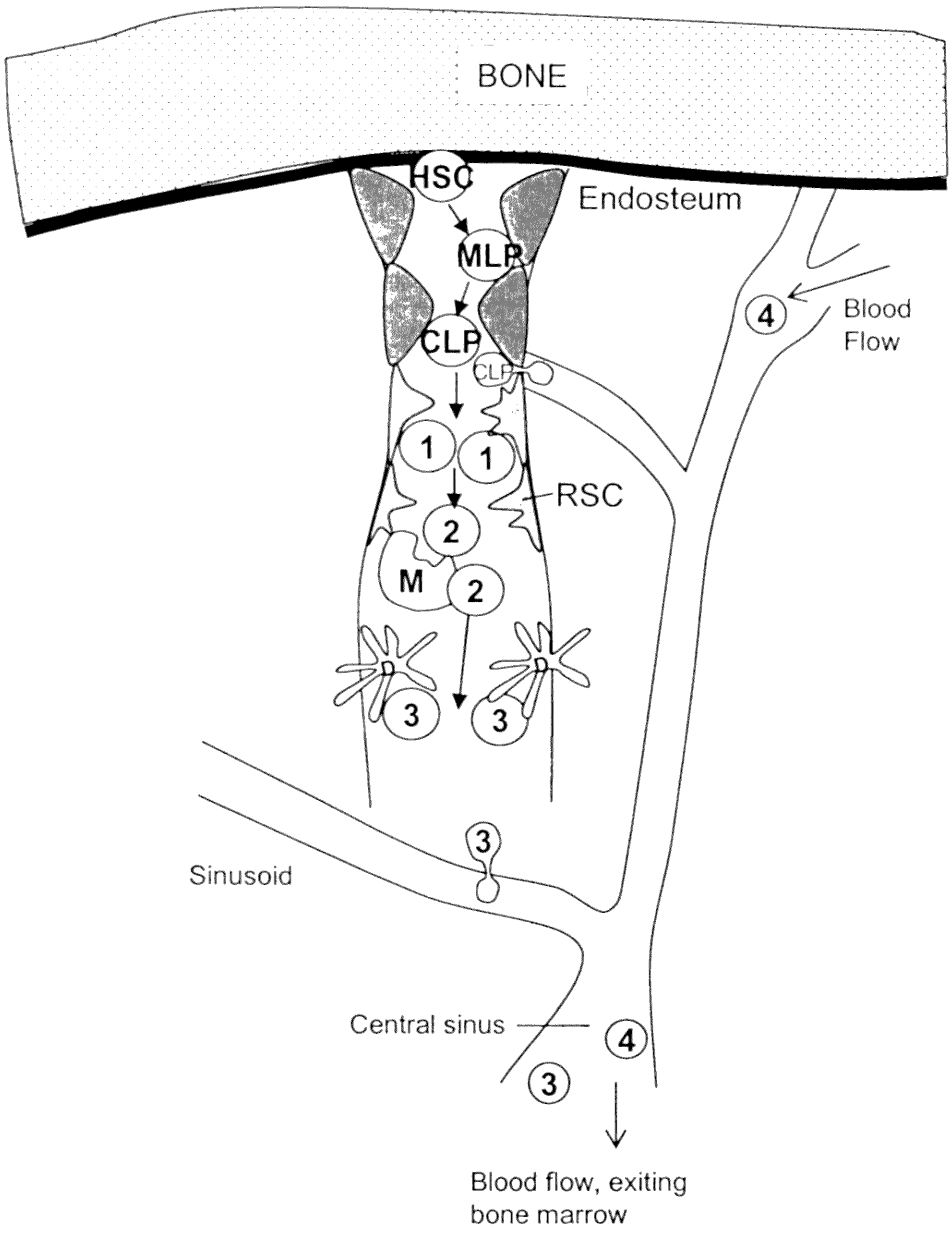

Figure 1.2. Schematic diagram of haematopoiesis and B cell development in the bone marrow.

HSC, haematopoietic stem cells. MLP, myeloid lymphoid progenitor. CLP, common lymphoid progenitor. M, macrophage. D, Dendritic cell. RSC, reticular stromal cells. 1, Pro-B cells. 2, Pre-B cells. 3, Immature B cells. 4, Mature recirculating B cells. ⟶ Differentiation.

The next stage in B cell development is the pre-B cell stage. This stage can be divided into two separate stages. After successful rearrangement, the heavy chain is expressed on the cell surface as part of the pre-B cell receptor together with a surrogate light chain. BLNK helps facilitate signalling through the pre-BCR via syk-kinase (16, 17). During the first pre-B cell stage, cells proliferate extensively, a process that is facilitated by the transcription factor LEF-1 (18). The pre-B cell then returns to a stable resting state and starts to rearrange the κ light chain. The V-J gene segments are recombined by somatic recombination as with the heavy chain. If this is not successful recombination repeated until either it is successful or there are no more J gene segments to rearrange. If this is still unsuccessful, the cell starts λ light chain gene rearrangement. As with the κ chain, the gene segments are recombined until there is either a successful in frame somatic recombination or the cells undergo apoptosis.

Once the cell has successfully rearranged its heavy and light chains, these are expressed on the cell surface as sIgM. The immature B cell now undergoes negative selection. All B cells that recognise cell-surface self molecules are deleted, and those B cells that recognise soluble self molecules are rendered anergic. The latter still migrate to the periphery and mature but are soon out-competed and die. These mechanisms prevent autoimmunity. The OCT-2 transcription factor is thought to facilitate the selection and maturation of the immature B cells by acting with the BCR (19).

Once immature B cells leave the bone marrow and enter the spleen they are referred to as transitional T1 B cells. These cells express the phenotype: $CD19^{+}$,

sIgM$^+$, CD21$^-$ and CD23$^-$. These T1 B cells develop successively into T2 B cells and then mature follicular B cells, which are CD21$^+$ CD23$^+$, and found in the follicular area of the spleen, or marginal zone B cells, which are CD21$^+$ CD23$^-$ (20). However the majority of B cells do not appear to fully mature, presumably because they are competing for space with mature B cells already present.

1.1.3.2. Marginal zone B cells

Marginal zone B (MZB) cells are found in the marginal zone area of the spleen, between the B and T cell areas and develop from the T2 B cells. It is thought T2 B cells develop into marginal zone B cells rather than follicular B cells through the activation of Notch2, although a characteristic of MZB cells is that their BCR recognises more innate motifs than random adaptive variation would predict (21) (20). Notably, B1 cells also mainly recognise more innate motifs, and MZB cells also share other characteristics with B1 cells.

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1.1.3.3. B-1 B cells.

B-1 B cells were originally thought to develop primarily from precursors among foetal liver cells, due to limited reconstitution after bone marrow transfer into irradiated recipients (22). They can be divided into two groups based of surface CD5 staining: B-1a CD5$^+$ and B-1b CD5$^-$ cells. It is thought that during the pro-B cell stage the CD5$^+$ B-1 cells may undergo positive selection via strong BCR signals. The B-1 cells constitutively express IL-5 receptor, unlike the B-2 cells (23). B1 B cells leave the bone marrow early in their development and migrate either to the spleen or the peritoneal cavity (24, 25). They have been found to secrete

antibodies without germline immunoglobulin rearrangement, and they do not always express the enzyme TdT (26-28). High levels of B-1 cells are found in the peritoneal cavity are a major source of autoimmune and anti-carbohydrate (innate) antibodies. These anti-carbohydrate antibodies typically recognise the same motifs targeted by the innate immune system, such as lipopolysaccaride (LPS) and are referred to as natural IgM antibodies.

1.1.4. Development of T cells

The common lymphoid progenitor can potentially differentiate into T, B or NK cell lineages. When it enters the thymus it is not clear whether it does so as a lymphoid progenitor or has committed to the T cell lineage developmental pathway (Figure 1.3). The progenitor is first recognisable as a double negative thymocyte when it enters from the blood stream to the medulla near the cortico-medullary junction (29). If the T cell develops into an $\alpha\beta$ T cell, then the DN thymocyte migrates to the sub-capsular zone of the thymus, but if the T cell forms a $\gamma\delta$ T cell, then the DN thymocyte migrates to the centre of the cortex. The mechanism determining $\alpha\beta$ vs. $\gamma\delta$ T cell differentiation is unknown. One thought is that if the TCR β chain has successfully rearranged before the γ and δ chains then the cell will continue on and rearrange the α chain to become an $\alpha\beta$ T cell. However, if the γ and δ chains are successfully rearranged before the TCR β chain, then the cell becomes a $\gamma\delta$ T cell. Another hypothesis is that notch activity favours the development of TCR $\alpha\beta$ T cells.

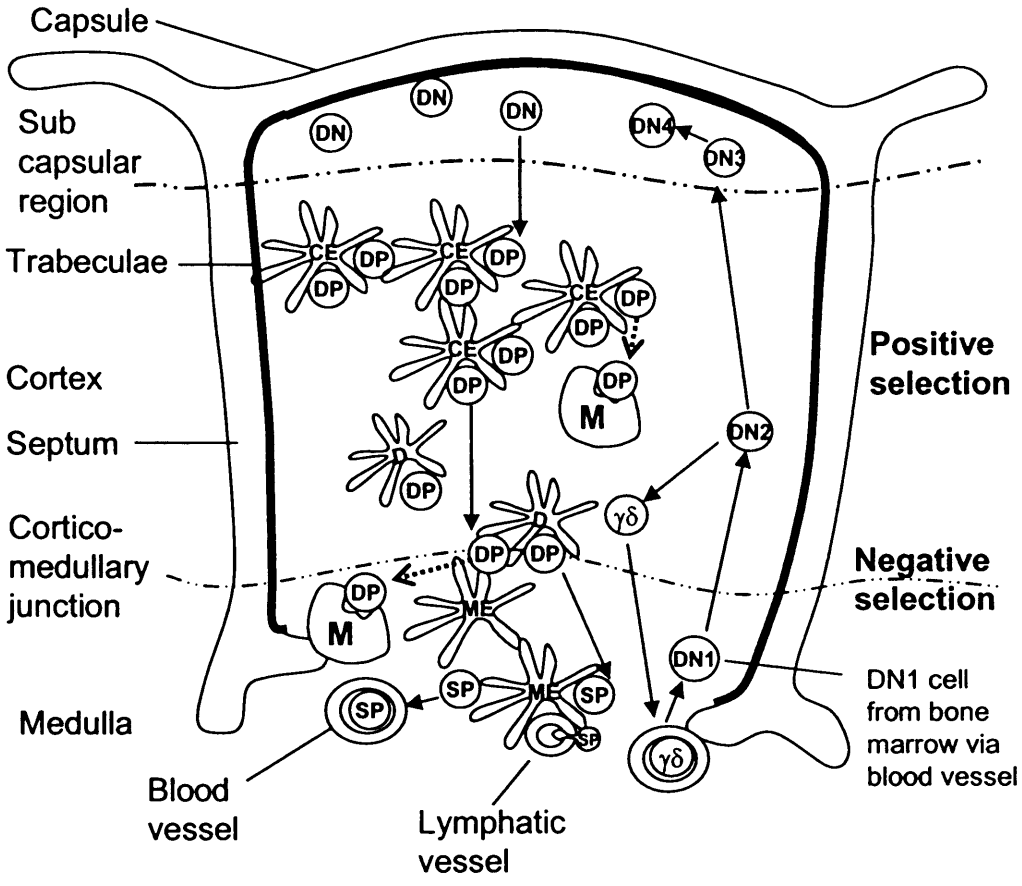

Figure 1.3. Schematic diagram of T cell development.

DN, Double negative T cell. DP, double positive T cell. SP, single positive αβ T cell. γδ, γδ T cell. M, macrophage. D, DC. CE, cortical epithelial cell. ME, medullary epithelial cell. ⟶ Differentiation ·····➔ Apoptosis and phagocytosis.

It is thought that TCR γδ signals may block notch activity and therefore prevent αβ gene rearrangement, although TCR signals are still necessary for survival (30-33). αβ T cells can be broadly divided into four thymic populations based on expression of the cell surface markers CD4 and CD8. The first or double negative stage (DN, $CD4^-CD8^-$), is followed during maturation by the double positive stage (DP, $CD4^+CD8^+$). The most mature thymocytes are the single positive (SP) populations: CD4SP ($CD4^+CD8^-$), or CD8SP ($CD4^-CD8^+$). The DN stage can be divided into four further stages depending on CD44 and CD25 expression: DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$), and DN4 ($CD44^-CD25^-$). The transcription factors Notch and Ikaros are essential for T cell development.

During the DN1 stage the T cell begins recombination of the TCR δ chain with recombination between the D-V gene segments. As with the B cells, both RAG proteins and TdT are present to provide genetic variation in the generation of the TCR (34). During the DN2 stage recombination of the γ chain starts. It is at this stage that the γδ T cells diverge from the αβ T cell lineage. In the DN3 stage recombination between the D-J gene segments on TCR β gene occurs. At the DN4 stage, the TCR β chain rearranges the V-DJ chain segments. Once the TCR β chain has rearranged, the cell is committed to becoming an αβ T cell.

Subsequently, the TCR β chain is expressed on the cell surface with a surrogate TCR α chain. Signalling through the pre-TCR prevents further gene rearrangements and initiates proliferation.

The cell then enters the double positive stage, expressing both CD4 and CD8 on the cell surface, and proliferating extensively. During this stage any TCR δ genes

remaining in the TCR α gene are removed through V-J TCR α chain gene rearrangements. Since there are multiple V and J gene segments, multiple rearrangements can occur to provide a successful rearrangement. The rearranged TCR α chain is then expressed on the cell surface with the TCR β to form an αβ TCR. Signalling through the TCR turns off the gene rearrangement machinery such as the RAGs and TdT. It has also been found that Notch is expressed in double positive thymocytes and Notch inhibits E2A transcriptional activation (35). thus also preventing further gene manipulation.

After the TCR is expressed on the cell surface, a process of selection, based on TCR specificity occurs. This is necessary to (a) delete all unfunctional T cells that can not recognise MHC, and (b) delete all T cells that could potentially become activated by and therefore attack self. First, cells that have produced a TCR that can recognise MHCs present on the thymic stroma are positively selected. Cells unable to recognise the MHC fail to receive a survaival signal and undergo apoptosis. However, cells expressing TCRs that bind the MHC too strongly receive a pro-death signal and also undergo apoptosis. The actual mechanisms for this are not fully understood, but the different outcomes could reflect either qualitative or quantitative differences in signalling. It is thought to be either a combination of affinity and avidity or differential signalling.

The last choice for the T cell to make is whether it develops from the double positive into either a $CD4^+$ or $CD8^+$ T cell. This decision is thought result from the nature of signal delivered through the TCR. It has been proposed that weak signalling, usually through MHCI leads to $CD8^+$ T cells, while strong signalling, mostly through binding to MHCII, leads to the development of $CD4^+$ T cells (36).

This process is also thought to involve notch, for notch1 can regulate TCR signal strength and CD4 vs. CD8 T cell development by modulating MAPK kinase, which is part of the differential signalling cascade (33, 37). Increased notch activity has been shown to promote CD8 T cell development.

Once the T cell has developed into either a $CD4^+$ or $CD8^+$ T cell, it is actively released into the periphery, where it re-circulates between the blood and the lymphoid tissues.

1.1.5. Development of other cell types

1.1.5.1. Natural Killer cells (NK)

NK cells have an innate ability to lyse cells that they recognise as targets, and produce cytokines, with which they regulate the local immune response. NK cells are thought to develop from the T cell progenitor in the bone marrow, and do not need to enter the thymus to mature. The cytokine IL-15 and lymphotoxin are essential for NK cell development and survival. The transcription factors Ets-1, Id2, Ikaros are essential for the development of NK cells from the T cell progenitor. (38-42).

NKT cells are T cells that posses NK cell markers. NKT cells have αβ TCR rearrangement, and express an invariant TCRα chain, but also express inhibitory MHC-specific NK receptors. They are auto-reactive and recognise self-peptides, but instead of being deleted in the thymus they are selected for. NKT cells

possess a high frequency of the specific Vα14-Jα18 gene rearrangement. NKT cells are also thought to regulate T cell responses. A disruption in NKT cell functions frequently leads to auto-immunity and tumour development. It is not known at what stage they diverge from the conventional T cell lineage, although they express both rearranged TCR α and β chains. It is not thought that they enter the double positive stage, but they have been found both in the CD4 single positive and the double negative stages. However, it has been observed that intra-thymically injected DP T cells can develop into NKT cells (43-46).

1.1.5.2. Dendritic cells (DCs)

Although the precise developmental pathways of DCs remain poorly understood, it appears that these cells can be derived from the myeloid lineage as well as the lymphoid lineage. Myeloid lineage DCs can be generated from lineage-committed macrophages and granulocytes, whereas lymphoid derived DCs can apparently be produced from both DN1 thymocytes and pre-B cells (47-51). Immature DCs are present in peripheral tissues and in lymphoid organs and are involved in immune surveillance, sampling the environment for foreign and self-antigens.

1.2. The immune response.

T cells become specifically activated via the TCR by peptide presented by the MHC. MHC class 1 (MHCI) presents peptides to $CD8^+$ T cells, whereas MHC class 2 (MHCII) presents antigen to $CD4^+$ cells. Antigen is taken up by antigen

presenting cells, APC's, the most efficient being the DC. The APC then migrates to the T cell area of secondary lymphoid tissue such as the lymph node and presents the antigen to naïve T cells. A specific interaction with the T cell results in the T cell becoming activated, clonaly expanding and producing effector cells to fight pathogens, either directly in the case of $CD8^+$ T cells or indirectly by $CD4^+$ T cells by supporting the B cell response.

This next section is divided into three sections the initiation of the immune response, the T and B cell responses, and the contraction and memory stage of the immune response.

1.2.1. The initiation of the immune response

Initiation of immune responses requires that APCs receive an activation signal. DCs become activated by either inflammatory signals such as type-1 IFN, or by the engagement of its Toll-like receptors (TLRs) or other innate receptors. TLRs recognise pathogen components such as LPS and double stranded RNA. DC activation triggers a series of events that lead to the stimulation of antigen-specific T cells (see below). Central to T cell stimulation is the processing and presentation of antigens (52).

1.2.1.1. Antigen uptake and processing

DCs continuously sample their environment, taking up moleculaes by pinocytosis, endocytosis or phagocytosis. After uptake, antigens are processed for presentation of peptides on MHCI or MHCII.

Different pathways are involved in the processing of antigens for presentation on MHCI and MHCII.

1.2.1.2. MHCI processing

Newly synthesised cellular proteins are used to generate MHCI peptides. They are approximately 8-10 amino acids long, and generated by proteolysis of cytosolic proteins by the proteosomes. The peptides are transported to the endoplasmic reticulum (ER) by the transport associated with antigen processing molecule or TAP, (53). After post-translational modification, the MHCI molecule associates with TAP and is assembled with the transported peptide present in its binding groove (54).

In addition to presenting peptide derived from newly transcribed proteins, MHCI molecules can also present peptides from internalised antigens through a prossess known as cross-presentation. Unlike traditional MHCI presentation, which all cells can carry out, cross-presentation is thought to be unique to APCs. Cross-presentation appears to involve fusion between phagosomes (containing internalised antigens) and the endoplasmic reticulum or access of soluble antigens to the endoplasmic reticulum, and requires the same machinery as that necessary for conventional MHCI presentation (55-61).

1.2.1.3. MHCII processing

MHCII molecules bind peptides that are between 13-24 amino acids long and are generated from proteins present in endocytic vesicles. The majority of proteins present in endocytic vesicles have been endocytosed from outside the cell (62).

However some intracellular proteins have been found to enter the endocytic vesicles (63, 64). Whilst in the endocytic vesicles, the proteins are denatured and degraded through a series of chemical reactions, until peptide fragments are produced. The peptide binds the assembled MHCII molecule in more 'mature', endocytic vesicles when all the denaturing has been completed and the peptide fragments have been generated. Newly assembled MHCII molecules associate with a molecule called the invariant chain. Association between the MHCII complex and the invariant chain, facilitates the transport of the MHCII complex out of the ER (65, 66). A segment of then invariant chain, class II MHC-associated invariant chain peptide (CLIP), binds to the peptide binding groove and stabilises the molecule. Disassociation of CLIP from the MHCII complex in late endosomes is followed rapidly by the binding of the degraded peptide. This complex is then transported and expressed on the cell surface. MHCII presentation is thought to occur in APC's such as macrophages, DCs and certain subsets of B cells.

1.2.1.4. Maturation and migration of the APC

Receipt of activation signals causes DCs to mature. As the DC matures, their function and phenotype changes. Pinocytosis is down regulated, and the cells express a different set of molecules controlling migration. For example CCR7 is upregulated, allowing DCs to migrate out of peripheral tissues and into the T cell areas of secondary lymphoid organs (67). Inaddition, DCs upregulate co-stimulatory molecules such as CD80 and CD86 which bind CD28 present on the naïve T cell, and express a range of chemokines and cytokines.

1.2.2. B cell response

1.2.2.1. T-independent B cell response.

This response produces a rapid expansion of antibody producing B cells, which lead to the formation of plasma cells. The T-independent response usually consists of either B-1 cells and/or marginal zone B cells. The T-independent response can be divided into two categories: TI-1, which is triggered by the antigens that can polyclonally activate the B cell without BCR signalling, or TI-2, which requires BCR activation and co-stimulation, though this can be provided by the APC (68-70). The antigens which trigger TI-1 responses are usually, commonly associated with pathogens, such as capsular polysaccharide from bacterial cell walls or repetitive viral antigenic epitopes. Once activated, cells responding to TI-2 antigens rapidly turn into plasma cells and secrete vast amounts of antibody. The responding B cells are mainly found in areas where there is a high potential for pathogenic encounters such as the intestine or the peritoneal cavity. In addition, the marginal zone B cells can also respond efficiently if the pathogens are present in the blood. This immediate production of antibodies could potentially delay the infection and allow the T cells and B2 cells to respond (71-73).

1.2.2.2. T dependent B cell response

The B cell recognises its antigen via the BCR, which consists of numerous Ig chains including the newly rearranged specific IgM, with an Igα and Igβ (CD79 a & b) complex. The B cell synapse, like the T cell synapse, is supported by rafts to

increase the stability and focus the signalling molecules into close proximity, this is stability is induced within seconds of BCR signalling (74).

1.2.2.3. The initiation and the B cell response.

As described above, the antigen is brought to the lymphoid organ such as the lymph node by the dendritic cell. After presentation of antigen to the $CD4^{+}$T helper cell, the T_H cell is activated and clonaly expands, and then migrates to the T/B cell zone border. At the same time B cells recognise intact, cellular antigen. The antigen binds to the BCR, triggering signalling and the internalisation of the antigen-BCR complex. The naïve B cell degrades and processes the antigen and presents it on its MHCII. The B cell then migrates to the T/B cell zone border, where it presents the antigen to the clonaly expanded T_H cell to receive T cell help. This is provided in the form of cytokines and CD40L. The activated B cells then either remains in the T/B cell zone border and becomes a short lived clonal plasma cell secreting antibodies, or it returns to the B cell area and to form germinal centres.

Isotype switching has been shown to be influenced by the T_H cell, via the cytokines that it produces, and signalling through CD40. CD40 signalling and cytokine signalling induce germline recombination so as to produce different Ig molecules. Plasma cells are terminally differentiated. They down regulate most cell surface molecules, only produce antibodies, and die within a few days.

B cells which form germinal centres in the B cell area of the secondary lymphoid tissue undergo massive clonal expansion to form this structure. Further T cell help within the GC is then required for somatic mutation. Somatic mutation involves

substitution, and occasionally insertions and deletions in the variable regions of the Ig gene (75). These mutations are generally thought to be carried out by an error prone DNA polymerase (76). These mutations allow the B cells response to produce antibodies with high affinity for antigen, which are selected from the population of cells expressing mutated receptors. Cells produced from the germinal centres can form plasma cells or memory B cells. Plasma cells formed from germinal centre B cells preferentially migrate to the bone marrow (77).

1.2.2.4. Antibody isotypes and functions

Antibodies bind to their target antigen, which results in numerous effects, depending on the type of antibody bound.

1.2.2.4.1. Neutralising Antibodies

These antibodies bind toxins present in the extracellular fluid, such as bacterial and snake toxins. Specific high affinity antibodies bind the toxin and therefore blocks its action. It is also possible for the high affinity antibodies to prevent viral infection by binding to free virus and preventing its binding to the cellular receptor.
Some of the processes that can eliminate these antigen-antibody complexes are described below.

1.2.2.4.2. Opsonisation

This occurs when antibodies bind to antigens such as bacteria, and then bind to phagocytic cells such as neutrophils. Binding to the cell triggers the neutrophil to engulf the antigen-antibody complex and destroy it, thereby killing the bacteria.

1.2.2.4.3. Activation of Phagocytes

In addition to inducing phagocytosis, antigen-antibody complexes bound to the surface of phagocytes can trigger cellular activation. Activation leads to the production of toxic moleculaes that can kill the pathogen.

1.2.2.4.4. Activation of NK cells.

Antigens present on the cell surface, such as viral proteins expressed by a virally infected cell, can be bound by high-specificity antibodies. These can activate NK cells to kill the infected cell or bacteria by antibody dependent cell mediated cytotoxicity.

1.2.2.4.5. Activation of complement

Specific antibodies bound to antigen can activate the complement cascade. This is a series of enzymatic reactions, that result in the production of a membrane attack complex, which will destroy the cell bound to the antibody. Alternatively, deposition of specialised complement proteins procide a signal for the cell to be opsonised, or cause inflammation resulting in the recruitment of phagocytotic cells.

1.2.2.4.6. IgA

IgA is associated with mucosal sites, and is transported across the epithelial cells into the lumen. IgA binds to invading pathogens, preventing their entry though the mucosal walls. The majority of the plasma B cells secreting IgA in the intestine appear to be derived from B-1 cells.

1.2.2.4.7. IgD

The function of IgD is unknown. sIgD is typically used to describe different stages in B cell maturation.

1.2.2.4.8. IgE

IgE the rarest of antibodies, is thought to be important in combating large parasites and is implicated in allergic reactions. It binds to mast cells and esoinophils. Binding and cross-linking triggers de-granulation, leading to secretion of inflammatory cytokines such as histamine, which in turn facilitates the recruitment of other immune cells.

1.2.2.4.9. IgG

IgG includes different sub-types such as IgG_1, IgG_{2a} IgG_{2b} and IgG_3 (in mice). The different sub-types can bind complement with different affinities, trigger opsonisation, and mediate NK cell cytotoxicity. IgG antibodies can be of high affinity and can act as neutralising antibodies.

1.2.2.4.10. IgM

IgM mainly activates the complement pathway, though it can also mediate opsonisation. In addition been shown to have neutralising activity.

1.2.3. T cell response

1.2.3.1. Activation of the T cell response.

The naïve T cell first encounters antigen in the peripheral lymphoid tissues such as the lymph node. Naïve T cells are generally found in the T cell area of the secondary lymphoid tissue. T cells enter LN due to the adhesion molecules present on the cell surface, which bind specific molecules on the lymph node blood vessels. Naive T cells use CD62L, CCR7 and CD11a/CD18 for entry into the LN,

via the high endothelial venules (HEV) (78). If a naïve T cell recognises the antigen presented by the DC, and there is sufficient co-stimulation available, then the T cell is primed.

For a naïve T cell to be efficiently activated it is thought that 3 signals are required: 1, a specific TCR-MHC complex; 2, co-stimulation from the APC; 3, cytokines providing survival and/or proliferative signals.

A high affinity TCR-MHC-peptide interaction activates downstream signalling events in the T cell, involving the intracellular signalling molecule Lck. Subsequently a signalling cascade results in the activation of lymphocyte genes involved in the immune response (79, 80). The extent of Tcell division is thought to depend on a multitude of factors including the availability of the peptide antigen. If only low amounts of antigen are present, this is thought to limit the TCR-APC interactions and therefore the size of T cell response (81). If co-stimulation, provided by the APC, is absent, functional activation of the T cell is prevented (82). One of the ways in which co-stimulation is thought to enhance T cell activation is by increasing the responsiveness of T cells to growth factors, such as IL-2 (83). Co-stimulation is also thought to stabilise the interaction between the T cell and the APC, increasing the binding time between the TCR and the MHC and recruiting more intracellular signalling molecules to the TCR.

Cytokines act on the T cell to provide a multitude of signals, including those regulating proliferation and survival. The magnitude of the response is greatly affected by the amount of inflammation and inflammatory cytokines, such as TNF-

α, present at the initiation of the immune response. Inflammatory cytokines increase the co-stimulatory molecules on the APC and induce migration into the T cell areas. Therefore, more inflammation leads to more DCs available to present antigen and grater co-stimulation (84). Inflammatory cytokines can also act on T cell's directly, increasing their responsiveness to growth factors (85-87).

1.2.3.2. Raft and intracellular proteins.

Successful TCR stimulation and co-stimulation result in signalling cascades at a very concentrated area of the T cell membrane. It has been found that this area of intense activity, the immunological synapse, is more rigid than other parts of the plasma membrane. This is due to the presence of lipid rafts, which corresponds to a high concentration of insoluble glycosphingolipids and cholesterol in the plasma bi-layer (88, 89). It has been suggested that the formation of a tight immune synapse decreases the presence of negative signalling regulators such as CD45 by steric-hindrance (90).

Changes in the cells' morphology, and potentially lipid rafts movements, are carried out by the cytoskeleton. The cyoskeleton consists of a web of actin, which contracts to change the cells' morphological shape, that is essential in the formation of the immunlogical synapse (91-93). Other proteins are involved with the cytoskeleton such as the ERM family proteins. These are explained further in the next section.

1.2.3.3. The effector stage of the immune response

Once a cell becomes activated it undergoes multiple divisions and turns on numerous genes associated with effector activity. Activation also changes the adhesion profiles of the effector cells, allowing them to infiltrate non-lymphoid tissues. PSGL-1 is thought to allow entry into the skin, whilst integrin $\alpha_4\beta_7$ (LPAM-1) is used to enter into the mucosal tissue (94). At the site of infection there is usually inflammation. Inflammatory cytokines change the adhesion molecules present on the stroma of the capillaries and venules, permitting effector cells to the site of infection.

Effector CD4 cells are often separated into T_H1 or T_H2 subsets that differ both in their function and in the cytokines that they produce. For example, IFNγ is a characteristic T_H1 cytokine that activates macrophages and stimulates B cells to produce opsonizing IgG_{2a} antibody (95). T_H2 cells characteristically produce IL-4, which stimulates B cells to produce IgG_1 neutralising antibodies. The different types of effector cell are effective against different pathogens. T_H1 effectors are mainly used against intracellular bacteria, fungi and protozoa, whereas the T_H2 response is mainly concerned with eliminating helminth parasites and bacterial toxins. How the decision to generate a T_H1 vs. T_H2 $CD4^+$ T cell response is poorly understood. The cytokine environment present during T cell activation is likely to play an important role.

Cytotoxic $CD8^+$ T cells, or CTLs, once activated, divide and produce a multitude of different effector molecules including IFN-γ, granzyme and perforin, which enables them to directly kill a cell expressing a MHCI complex loaded with the specific peptide. Perforin released from the CTL punches holes in the membrane of the

target cell and destroying it. Serine proteases, such as granzyme, are produced in vesicles that fuse with the membrane of the target cell, thereby releasing the granzyme into the cytoplasm of the target cell and activating apoptosis. CTLs can also trigger apoptosis by ligating Fas on the target cell with Fas ligand on the $CD8^+$ cell. Fas initiates a signaling cascade resulting in the activation of caspases and apoptisis of the target cell. It has been reported that the CTLs can not produce IL-2, simultaneously with IFN-γ in sufficient quantities and therefore are dependent on the IL-2 produced by the $CD4^+$ T cell (96). CTLs generally target virally infected cells; by killing the cell, the virus is also destroyed.

1.2.4. Termination of the immune response and memory formation

Due to the limited space available in the body of an organism it is impractical for all the effector cells generated during an immune response to remain alive and viable. In fact, the vast majority of activated cells die after clearance of the pathogen. However, a propotion of the cells do survive and persist as memory cells. These cells provide a stronger and faster immune response should there be a re-infection. This is due to both the higher frequency of antigen specific cells among memory cells, and altered properties of memory cells on a per cell basis.

1.2.4.1. Memory B cells.

Memory B cells, like plasma cells, are generated in the germinal centre reaction (77). These cells have the capacity for rapid expansion and the formation of plasma cells if the specific antigen is re-encountered, with the help of the memory T_H cells. It is thought that memory B cells can act as APC's to stimulate the T_H cells, which in turn, can stimulate the memory B cell. In addition, to memory B cells, some plasma cells survive and produce antibodies long-term in the absence of antigen (97).

1.2.4.2. T cell memory

1.2.4.2.1. $CD8^+$ memory cells

After the initial contraction of the effector cells, the frequency of $CD8^+$ memory cells remains constant. This is associated with a small amount of turnover, in which proliferation is balanced by death from the pool. Turnover is likely regulated by a number of factors, including contact with cytokines and specific cross-reactive antigen (98, 99). There are thought to be thought to be two types of $CD8^+$ memory cells, effector memory and central memory cells. Effector memory cells retain expression of effector genes, for example, perforin and granzyme, and are thought to circulate through the peripheral tissue (67, 100). Central memory cells do not have stores of cytotoxic enzymes and re-circulate between the blood and lymphoid tissues, much like naïve $CD8^+$ T cells.

1.2.4.2.2. $CD4^+$ T memory cells

Unlike $CD8^+$ memory cells, $CD4^+$ memory T cells appear to gradually decrease in number over time (101-103). These different kinetics likely reflect different requirements for survival and proliferation (104). As for with CD8 memory cells, $CD4^+$ memory cells can be divided into effector and central memory populations (105).

1.3.CD44

CD44 is an 85KDa transmembrane glycoprotein, which is expressed on numerous cell types including epithelial cells and most haematopoietic cells. CD44 has restricted expression in embryogenesis including the mesoderm layer (106). It has been implicated in numerous processes including cell adhesion, lymphocyte migration and activation, embryogenesis and tumour metastasis (107-113). CD44 has a complex gene structure, in which multiple isoforms can be generated. Interest in the function of different isoforms has stemmed largely from observations on tumour cells, where expression of certain variant exons of CD44 has been shown to correlate with tumour progression and the expression or absence of certain isoforms have a certain amount of prognostic value. However, CD44 appears to mediate important functions on T cells, as both activated and memory T cells express high levels of CD44 on the cell surface. Nevertheless, the pattern of variant CD44 exon expression on normal T cells and how they influence cellular

function remains poorly understood. Below is a brief review of the properties of CD44 and it possible functions on various cell types.

1.3.1. CD44 gene structure and homology

The murine CD44 gene is found on chromosome 2 close to the RAG genes. It consists of 20 exons, 10 of which can be alternatively spliced (v1-v10) potentially generating over 1000 isoforms (Figure 1.4). The most common form of CD44, otherwise known, as the standard isoform (CD44s), is the lowest molecular weight form at 85KDa of this molecule and contains no variant exons. The variable exons are inserted near to the plasma membrane on the extracellular side on the molecule (Figure 1.5). These variant exons add extra functions to the CD44 molecule, e.g. v3 contains a heparin binding site, which facilitates binding of the heparin binding growth factor. The variant exons are of interest to cancer biologists, for the presence of certain variant CD44 exons has been used as a prognostic maker for particular types of tumours.

Diversity of the CD44 molecule is also generated at the post-translational level, for the mature protein contains multiple glycosylation sites (114). Therefore the RNA and protein diversity exhibited by the CD44 molecule can generate a huge variety of CD44 isoforms, although most of these isoforms have not been detected on cells.

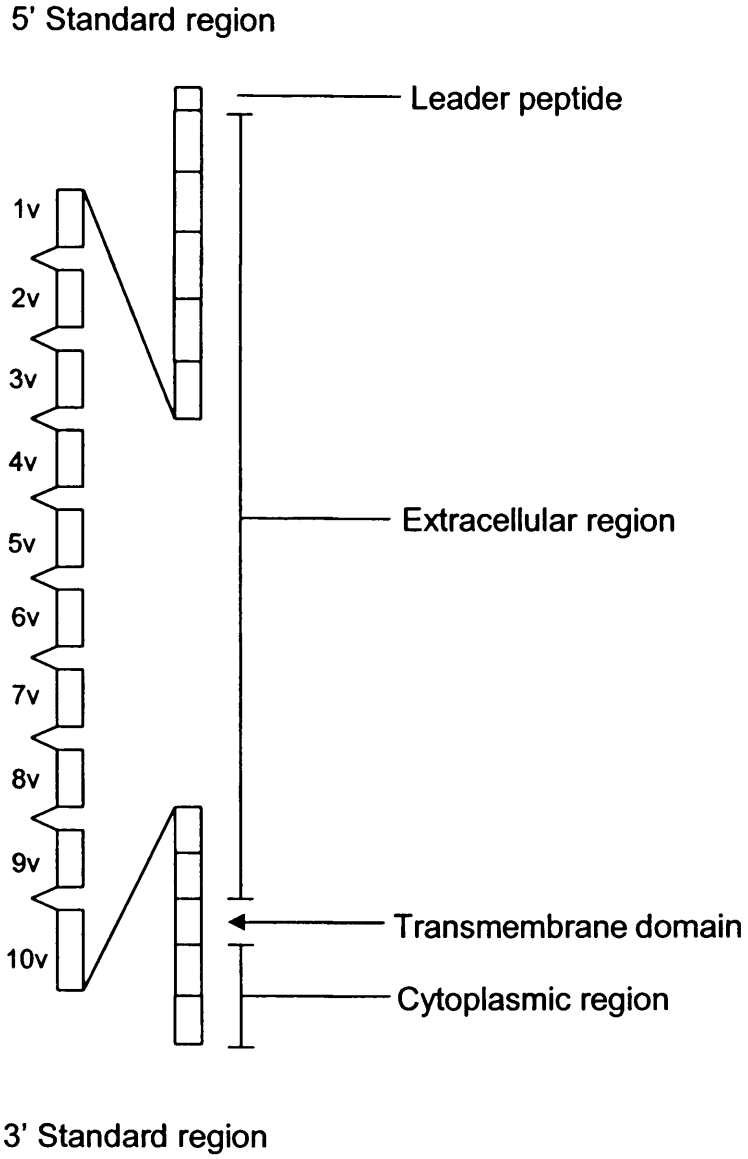

Figure 1.4. Diagram of murine CD44 genomic structure.

A schematic diagram of the murine CD44 gene structure, showing the orientation of the 10 variable exons (v). This diagram also shows the exons present in the extracellular, transmembrane and intracellular domains. The exon lengths are not to scale.

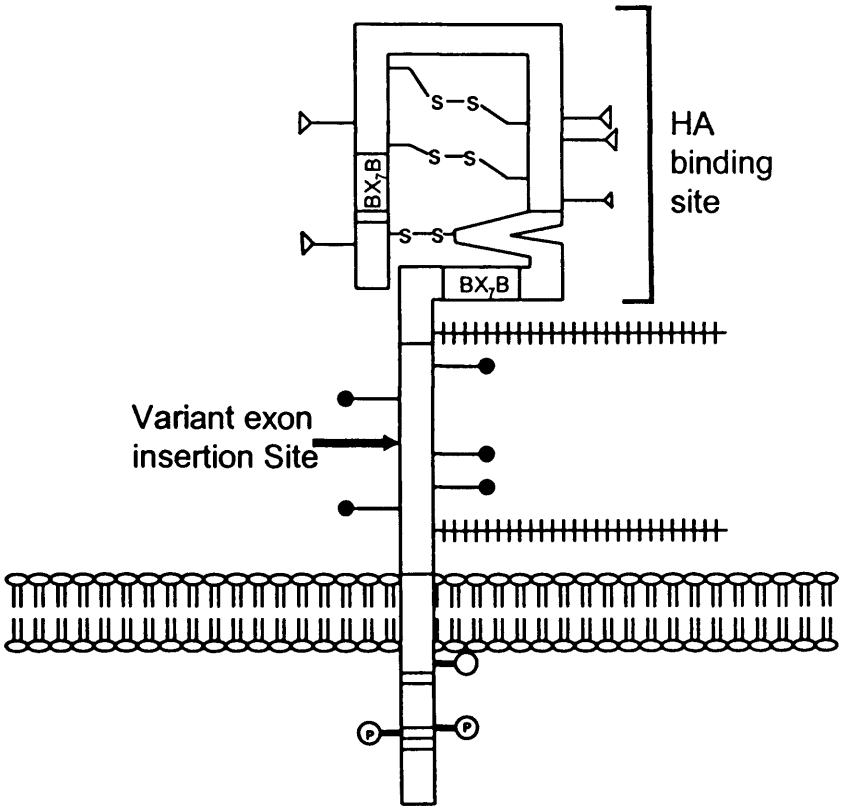

Figure 1.5. Diagram of tertiary structure of CD44.

—s— Potential disulphide bonds

—◁ Potential sites of N-Linked glycosylation

—Ⓟ Potential serine phosphorylation site

—● Potential O-linked glycosylation

HHH Potential sites of GAG addition

The extracellular region of CD44 has 85% homology amongst mammalian species. It contains six cysteine residues, which have the potential to form disulphide bonds, as well as five conserved N-glycosylation sites.

A non-conserved region flanks the variant exon region, which shows 35% homology between mammalian species and contains potential sites for multiple carbohydrate modifications. The membrane proximal domain has 50% homology between mouse and man, while the transmembrane domain has 100% homology between mammalian species; the latter is required for dimerization of CD44 molecules, which has been shown to increase hyaluronic acid (HA) binding (HA is one of the major ligands for CD44, see below) (115). The cytoplasmic domain of CD44 has 85% homology between mammalian species. It does not seem to be required for extracellular ligand binding, although it has been suggested that it is needed to aggregate CD44 in clusters at the cell surface and therefore facilitate ligand binding. Some molecules of CD44 are phosphorylated on their serine residues in the cytoplasmic region. The cytoplasmic domain also contains binding site for tyrosine kinase and other signalling molecules.

1.3.2. Function of CD44

Multiple functions have been attributed to CD44. In general though, CD44 seems to be involved in cell movement, by cell-to-cell or cell-to-matrix interactions, and cellular activation, by its association with the intracellular cytoskeleton and src kinases. The function of variant exons has mainly been determined by antibodies

specific for the variant regions, although these do not always bind due to variable post translational modification (116).

1.3.2.1. Adhesion and cell migration.

CD44 binds to a number of molecules in the extracellular matrix, with its principle ligand being HA. Not surprisingly, therefore, an important function of CD44 is thought to be as an adhesion molecule. On T cells, a variety of adhesion molecules play essential roles in regulating cell migration and activation. When T cells become activated, they acquire the ability to migrate into sites of inflammation. Upregulation of CD44 on activated T cells implies that this molecule is involved in migration into tissues, and there is some evidence to support this idea. DCs also upregulate CD44 upon activation, which is associated with the expresion of v3, v6 and v9 (117). In fact, most haematopoietic cell upregulate CD44 in repose to inflammatory cytokines. Moreover, HA is produced by endothelial cells in response to TNFα and IL-1β, thereby facilitating the extravasation of activated immune cells into inflamed tissue (107, 118-120). Treatment of mice with anti-CD44 has been shown to inhibit the infiltration of inflammatory cells (121-124). Notably, some metatastic tumours which exhibit de-regulated migration, express CD44 variant exons.

Haematopoietic cells have been shown to interact with stromal cells via CD44. Disruption of these interactions leads to a marked reduction in T and B cell development and myelopoiesis (125, 126). The ligand on the stromal cells is not known (127). Variant exons of CD44 are expressed in early thymocytes, and

antibodies to these variant exons have been shown to block haematopoietic-stromal cell interactions (128).

1.3.2.1.1. Hyaluronic acid

The primary ligand of CD44s is the glysosaminoglycan (GAG), HA (129). HA is found as an integral part of the extracellular matrix. It acts by binding salt and water and expanding the extracellular space (130-136). CD44 binding to HA is seen in processes that involve cell-to-cell contact, adhesion, and transport through extracellular matrices.

Binding of cells to HA via CD44 is a highly regulated process. In fact, there are three states of HA binding: active, inducible and inactive. The active form of CD44 binds HA; this is the form of CD44 on some cancer cell lines and activated T cells. Inducible isoforms of CD44 can bind HA after activation. This form is expressed on naïve lymphocytes, and cytokines such as TNF-α, TGFβ and those signalling through the murine IL-2R γ–chain have been shown to induce activation of CD44 by post-translational mechanisms; conversely, IL-4, IL-10, and IL-13 inhibit this activation (136-139). Cells expressing the inactive form can not bind HA even after activation. The ability of inflammatory cytokines to enhance CD44 binding to extracellular matrix is in keeping with their role in recruiting cells of the immune system to sites of inflammation and infection. By corollary then, it would seem that regulation of the CD44 binding state is an important process regulating leukocyte migration. This regulation is quite complex, however, as cellular activation per se is insufficient to induce CD44 binding to HA. For example, HA adhesion is not stimulated by activation of B cells with LPS, pokeweed mitogen, or anti IgM, but is following treatment with PMA (140-144).

The CD44-HA interactions have been demonstrated in numerous models using various cell types. The importance of HA binding during lymphocyte extravasation has been shown using a rat model. In these experiments, intra-peritoneal injections of low molecular weight HA, (which should block CD44-HA interactions) in conjunction with cyclosporin treatment was found to prolong allograft survival compared to cyclosporin treatment alone. It was thought that the injected HA functioned by preventing the lymphocytes from entering the graft (145). In this respect, the importance of interactions with HA in mediating cell movement between tissues and the blood stream is also exemplified by the behaviour of tumour cells. In particular, tumours that produce HA are known to have a poor prognosis, presumably because they can use the HA as a scaffolding in metastasis.

Other cell types apart from leukocytes also express HA binding CD44. For example, platelets express the active HA-binding form of CD44. This contributes to the ability of platelets to respond readily to vascular injury, since HA is found immediately beneath the endothelial cells of many blood vessels in the dermis, the lamina propina of the intestine, the lungs, and pericardium. Thus, when these vessels are damaged, HA becomes exposed, allowing platelets to bind and initiate the repair process (146).

As discussed above, regulation of CD44 binding to HA is complex. The ability of CD44 to bind HA can be altered by post-translational modifications, dimerisation, and by the presence of certain variant exons, which can increase the binding ability of CD44 to HA (135). It has been found that v6 and v9 are essential for HA binding, while expression of CD44 v4-7 increases the ability of CD44 to bind

soluble HA (147, 148). Furthermore, binding to HA also depends on the cell type in which CD44 is expressed (149). This is likely due to the glycosylation competency of the cell, since both O- and N-linked glycosylation of the CD44 molecule changes its affinity for HA (114, 135, 150-152).

Another factor to take into consideration is the action of hyaluronidase. Hyaluronidase is an enzyme that degrades HA, and somehow modulates CD44 expression. It has been proposed that the CD44 isoforms, containing splice variants are unstable, and that they require the continuous presence of their ligand for stable expression: this could explain their transient expression (153). HA in the extracellular matrix is also continuously endocytosed and replaced, HARE (hyaluronic acid receptor for endocytosis) was found as an endocytic receptor for HA on liver sinusoidal epithelial cells (154), therefore HARE and RHAMM are two other cell surface proteins which can bind HA.

1.3.2.1.2. E-selectin

CD44 also binds to the adhesion molecule E-selectin. E-selectin is expressed on inflamed (cytokine or endotoxin stimulated) but not unstimulated endothelium, and is thought to play a role in mediating tissue specific homing of T cells into the skin and of primitive progenitor cells into the bone marrow. However, the major ligand of E-selectin on T cells is PSGL-1 rather than CD44. The CD44 found on the progenitor cells, which can bind E-selectin, has sialyated and fucosylated binding determinants on the N-glycans; this is not found on mature haematopoietic cells (155).

1.3.2.1.3. L-selectin, (CD62-L)

A sialofucosylated glycoform of CD44 expressed on human haematopoietic cells binds L-selectin. L- selectin is mainly expressed on lymphocytes and is involved in the rolling of lymphocytes to the endothelial cells. It is also mediates trafficking of lymphocytes to lymph nodes. In this report by Dimitroff *et al*, L-selectin was shown to be also expressed on human haematopoietic cells, hence in this situation, promote CD44 appears to promote homotypic interactions (156).

1.3.2.1.4. Chondritin Sulphate A, (CS-A)

CS-A along with HA accounts for the majority of extracellular matrix components in the bone marrow (125). CS-A has been shown to bind to CD44v expressed by myeloid progenitors, implying that CD44 may be involved in the migration of these cells in the bone marrow. v10 promotes cellular adhesion through recognition of chondritin sulfate (157)

1.3.2.1.5. Aggrecan

Aggrecan, is a chondrition sulphate proteoglycan and a major component of cartilage. CD44 has been found to bind to aggrecan side chains independently of HA, in a similar manner to that of chondriton sulphate A (158).

1.3.2.1.6. Chondritin Sulphate form of Invariant chain

As discussed in the previous section, the Invariant chain (Ii) associates with MHCII (159). A small proportion of the MHCII molecules on antigen presenting cells (APC) has been modified by the addition of chondritin sulphate, (Ii-CS). The binding of CD44 on the T cell to the modified MHCII on DCs is thought to enhance the stimulation of the cells provided by the APCs.

1.3.2.1.7. Fibronectin

CD44 binds the extracellular matrix protein fibronectin. An insoluble form of fibronectin is found in the connective tissue. It is also present as a soluble plasma protein in the serum. Fibronectin can be attached to other extracellular matrix proteins such as fibrin, and collagen and GAG's (160, 161).

1.3.2.2. Morphological changes by cytoskeletal rearrangement

Different changes in cell morphology are observed when CD44 is bound by HA versus immobilised anti CD44 antibody (162). For example, monocytes have been shown to bind and spread when cultured on plate-bound anti-CD44, while they bound but did not spread when put on plates coated with HA. In the presence of HA, the cells remained small and actin was diffuse. In contrast, on the plate bound anti-CD44, the cells were enlarged and the actin was concentrated in cellular extensions. While this may reflect the much higher affinity of the anti-CD44, it also suggests that the binding of CD44 on the cell surface can produce different results. In nerve cells CD44 is only expressed in astrocyte projections, which suggests its involvement in the maintenance of a stable central nervous system cytoarchitecture (163). Bacteria have also manipulated the cytoskeletal arangement properties of epithelial cells and use CD44 as a receptor to induce cytoskeletal rearrangement to allow invasion (164)

1.3.2.2.1. Ezrin, radixin and moesin

Morophological changes induced by CD44 binding may be related to the ability of this molecule to associate with a number of molecules involved in cytoskeletal organisation. Three such molecules are ezrin, radixin and moesin, (ERM), which

are cytoplasmic proteins (165, 166). These proteins act as cross linkers between cortical actin filaments and the plasma membrane, and are involved in the formation of microvilli, cell adhesion sites and clefts and ruffles in the plasma membrane. ERM are activated via Rho signaling pathways, and it is thought that they link the actin filaments and CD44. There is also evidence that there is an increase in affinity for ERM binding when v9 and/or v10 are present in CD44, even though the cytoplasmic domain is the same as in CD44s. ERM mediate outside-in, not inside-out signaling of adhesive receptors (167). ERM proteins share a FERM domain (band four point one, ezrin, radixin moesin homlogy domain) with Merlin. Therefore, both the ERM and Merlin (see below) could potentially bind CD44 via the FERM domain. Due a 60% homology between the two FERM domain of Merlin and ERM, this could contribute to the differences seen in the binding of CD44 to either Merlin or the ERM proteins (168).

1.3.2.2.2. Merlin

Another cytoskeletal associated molecule to which the cytoplasmic portion of CD44 binds is Merlin, an ERM related protein. At high cell densities, merlin mediates cell growth inhibition in response to HA binding of CD44. Merlin seems to form the basis of a molecular switch that specifies whether cells continue to divide (169). Although CD44 can bind both ERM and Merlin, these complexes appear to have contrasting roles, as shown by studies of tumour metastasis. Thus, it is thought that CD44 binds ERM and promotes invasiveness, but that cell confluence leads to CD44 binding merlin and inhibition of tumour metastasis (170).

1.3.2.2.3. Ankaryin

CD44 also binds to ankaryin, which serves as an adapter between the plasma membrane and the cytoskeleton (171, 172). Ankaryin is thought to be involved in phagocytosis of bacteria, by CD44 linkage to cytoskeletal elements (173). CD44-ankarin binding is thought to be modulated by protein 4.1 (174, 175).

1.3.2.3. CD44 signalling

Studies have shown that in addition to acting as an adhesion molecule, CD44 triggering can result in the transmission of signals to the cell, and activate it. Many experiments have been carried out using monoclonal CD44 antibodies to investigate the consequences of CD44 signalling in different cell types. Administration of anti-CD44 antibodies to *in vitro* human NK cells was shown to result in a dose dependent enhancement in NK cell cytotoxicity against tumour cells (176). Furthermore, an importance for CD44 signalling in NK cell activity was suggested by the fact that NK cells from $CD44^{-/-}$ mice could not adhere properly to target cells and therefore could not kill them (177). In nerve cells, CD44 has been implicated in enhancing neuroregulin signalling by schwann cells through constitutive association with erbB2 and erbB3, which are receptor tyrosine kinases expressed in schwann cells. Blocking CD44 expression in schwann cells by sense and antisense oligonucleotides, resulted in a loss of signalling and an increase in apoptosis (178).

CD44 also acts on T cells as a co-stimulating molecules as well as an adhesion molecule. Co-stimulation of a T cell through CD44 leads to an increase in T cell proliferation. T cells that express rat v4-v7 (as well as their own normal CD44) had

an accelerated response when exposed to antigen. Whether this is a property of v4-v7 or the fact that there is more CD44 on the cell surface was not discussed (179). v6 and v7 have been implicated in T cell activation (180, 181). v7 seems to be important for survival of effector lymphocytes in the intestine, since experimentally induced colitis in mice could not be induced in v7 deficient mice, and administration of anti-v7 antibody led to complete remission of the colitis in control mice. Also, administration of anti-v7 antibody in another study led to a decrease in IL-12 production (180, 182, 183). v6 has also been implicated in cellular activation. Not only is v6 upregulated after T cell interaction, but experiments have shown that anti-v6 antibody can block lymphocyte activation and decrease IL-2 and IFN-γ production (182, 184).

CD44 is also associated with apoptosis as well as proliferation. It is thought that CD44 supports both apoptosis and proliferation by enhancing signal transduction via TCR-CD3 complexes. A low level of signalling through CD3 and CD44 cross-linking results in apoptosis but higher levels (still sub-proliferation levels) of CD3 signalling and CD44 cross-linking results in proliferation (185). The presence of HA can also act as a co-stimulator for activated human T cells (176). Furthermore, signalling through HA-CD44 interactions can also induce murine B cell activation (186).

1.3.2.3.1. Rafts

Rafts are organised by actin to create a firm platform for synapse interactions. Signalling through CD44 initiates cytoskeletal rearrangement and membrane reorganization; this involves GTPase Rac association. The rafts in which CD44 localizes have been shown to contain Annexin II. Annexin II is a calcium binding

protein that can associate with actin filaments and membranes and is a major cellular substrate for src-family of protein kinases, which are associated with lipid rafts (187, 188). Shigella protein IpaB binds CD44 and invades using increased concentration of rafts, probably due to CD44 signalling to the cytoskeleton (189).

1.3.2.3.2. Intracellular signalling

Signalling through CD44 also involves the tyrosine kinase molecules lck and fyn, molecules which co-localise with the TCR to initiate the signalling cascade. (185, 190). Signalling through $P56^{lcK}$ leads to ZAP-70 phosphorylation (same as for the TCR) and this leads to an association between ZAP-70 and the TCR (191). Signalling through CD44 also induces an interaction with Tiam1 which increases Rac-1 signalling. HA binding to v3 stimulates Tiam1 associated – rac1 signalling, while v3 binding of heparin sulphate binding growth factors also induces Rac-1 signalling. Cross-linking CD44 has been shown to lead to the same signalling cascades as those initiated by cross-linking CD3. This is likely related to the fact that CD44 has also been found to associate with CD4 and CD3 on the cell surface. In this respect, it is interesting to note that binding CD4 with gp120 (from HIV) potentiates CD44–HA adhesion (192).

Rac-1 GTP binding protein activates actin cytoskeletal rearrangement, during processes such as the production of lamellipodia and reorientation of the cells in the direction of a migratory stimulous (171, 193-195).

1.3.2.4. Receptor for chemotactic and growth factors

CD44 can bind certain growth factors and chemotactic factors. This property has been propsed to play a role in the ability of tumor cells, for expression of variant CD44 exons responds with an increasing responsiveness to more growth factors (196).

1.3.2.4.1. Osteopontin

CD44 has been shown to bind to certain chemotactic factors, that have been produced in response to infection. One of these is osteopontin (OPN), a chemotactic factor that is secreted by a number of cell types including activated T cells and macrophages and which induces macrophage migration. When OPN is secreted from activated T cells, it induces chemotaxis by specific binding to CD44 present on macrophages and T cells. OPN can augment IFN-γ and CD40 expression after anti-CD3 stimulation (197). It has been shown that OPN is produced as a result of IL-3 or GM-CSF signalling, and can subsequently bind to surface CD44 and promote the anti-apoptotic activities of IL-3 and GM-CSF (198). This might explain some studies that have shown that cells responding to osteopontin exhibit enhanced cell survival through inhibition of apoptosis. Osteopontin deficient mice have an impaired Type 1 immunity to viruses and bacterial infection. They exhibit decreased IFN-γ and IL-12 but an increased IL-10 production. The mice show decreased granuloma formation in response to polyvinylpyrrolidone. These mice also exhibit reduced experimental autoimmune encephalomyelitis, in which there was less inflammation and infiltration of inflammatory cells seen in the $OPN^{-/-}$ mice compared to the controls (199). These observations may be relevant to the metatastic proficiency of tumour cells with

elevated OPN expression (200-202). CD44 binding of OPN can inhibit IL-10 production in macrophages (203). OPN can also inhibit nitric oxide production in macrophages. This is part of a negative feed back loop to limit the production of inflammatory cytokines since OPN expression can be induced by nitric oxide (204).

1.3.2.4.2. Macrophage Inflammatory Protein-1β. (MIP-1β)

A second chemotactic factor to which CD44 has been shown to bind is macrophage inflammatory protein-1β (MIP-1β) (205). This cytokine induces both chemotaxis and adhesion of T cells to the endothelium. Binding of MIP-1β to CD44 has been shown to increase the ability of CD44s to bind HA. Whether it binds to both CD44 and HA directly to produce this effect, or binds to CD44 and induces a conformational change which results in an increased binding of CD44 to HA is not known (206).

1.3.2.4.3. Matrix Metalloprotein-9, MMP-9 (gelatinase B)

A proteolytic form of MMP-9 associates with CD44 on the surface of mouse carcinoma and human melanoma cells. CD44 associated cell surface MMP-9 promotes cell-mediated collagen IV degradation *in vitro*. It is thought that CD44 serves to anchor MMP-9 on the cell surface possibly explaining a mechanism for CD44-mediated tumour invasion (207). In this regard it is worth noting that MMP-9 deficient mice have suppressed experimental metastasis (208).

1.3.2.4.4. Chondritin-4-sulphated serglycin

The serglycins are a family of proteogylcan molecules which have a core protein in common (209). Their expression is restricted to the yolk sac, haematopoietic cells and some tumours. In haematopoietic cells the serglycins are found in secretory granules, where they are thought to act as scaffolding during packaging, and

chaperone the secreted serine proteases. Serglycin has also been implicated in the differentiation of myeloid cells. Chondritin-4-sulphated serglycin binds CD44 on the HA binding site, and provides a co-stimulatory signal when present with CD3 stimulation. Serglycin also enhances the release of granzyme A from cytotoxic T cells (158, 210).

1.3.2.4.5. Heparin binding growth factor.

It is thought that sCD44 can not bind heparin binding growth factor, but expression of v3, plus modification of CD44 by the addition of heparin sulphate induces CD44 binding to heparin-binding growth factors (113). Expression of v2-10 has been shown to confer a metastatic phenotype on tumour cells, probably due to the presence of the heparin sulphate attachment site on v3 (211).

The heparin sulphated proteoglycan v3 of CD44 on monocytes has also been demonstrated to bind macrophage derived growth factor FGF-2, vascular endothelial growth factor, and heparin binding epidermal growth factor (212).

1.3.2.5. Soluble CD44

As well as the normal transmembrane form of the protein, CD44 has also been found in the soluble form. Soluble CD44 is released from the cell surface through metaloproteinases or serine proteases, but CD44-shedding from adherent cells is controlled by protein kinase C and Rho GTPase through the cytoskeleton (213, 214). Naturally occurring soluble CD44 enhances HA binding of surface bound CD44 instead of blocking it. It is thought to act in this way by adhering to chondritin sulphate side chains which are attached to cell surface CD44, thus, generating a multivalent complex with increased avidity for HA (215). However, secretion of

soluble CD44 can be enhanced by PMA treatment with anti-CD44 in some cell types and this soluble CD44 contributes to anti-invasive and anti-angiogenic properties (216). CD44 shedding may be a mechanism that allows the cell to be released from its interaction with the extracellular matrix, although it is interesting to note that unbound as well as anti-CD44 bound molecules are released in response to the plate-bound antibody.

Soluble CD44 has been found in serum of cancer patients and is generally associated with a poor prognosis. Membrane type 1 matrix metalloproteinase (MT1-MMP) has been shown to cleave CD44 from the surface of the cell and promote cell migration. In this process MT1-MMP degrades the extracellular matrix at the pericellular region and acts as a processing enzyme by releasing CD44H into the medium, which then stimulates cell motility (217). However, it should be borne in mind that some experiments have shown that soluble CD44 inhibited melanoma binding to HA, and that co-transfection of cells with a soluble form of CD44 led to decreased tumorigenesis (218).

Statement of scientific objectives

The aim of this thesis is to investigate the function of CD44 on T and B lymphocytes. Radiation bone marrow chimeras will be generated to allow CD44-deficient and CD44-wild type cells to develop in the same environment, and the fate of the two cell types will be compared in steady state and in response to immunisation.

1. To investigate the role of CD44 in T cell development
2. To investigate the role of CD44 in B cell development
3. To investigate the contribution of CD44 to mature T and B cell function.

2. Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Mice

All strains were obtained from the specific pathogen free unit at the institute for animal health (Compton, UK). Some C57Bl/6 mice were purchased from Charles Rivers UK.

Strain	Description
C57Bl/6	H-2^b, CD45.2, CD229.2, $CD44^{+/+}$
Ly5.2	H-2^b, CD45.1, CD229.2, $CD44^{+/+}$
$CD44^{-/-}$	H-2^b, CD45.2, CD229.1, $CD44^{-/-}$
129Sv	H-2^b, CD45.2, CD229.1, $CD44^{+/+}$
Balb/c	H-2^k, $CD44^{+/+}$
2C	CD8 TCR transgenic recognising H-2Ld + LSPFPFDL or H-$2K^b$ + SIYRYYGL peptide (C57Bl/6 H-2^b background)
$RAG^{-/-}$ B6RA61	C57Bl/6 mice with a deficient RAG gene, therefore no mature T or B cells
OT2	CD4 TCR transgenic specific for OVA323-339 peptide + I-Ad. (RAGKO B6 H-2^b background)

Table 2.1: Table of mice used

2.1.2. Normal Reagents

Reagent	Supplier	Catalogue Number
Agar	BDH	20768292
Ammonium chloride	Sigma	A5666
Ammonium Sulphate	Sigma	A2939
Betaplate Scintillant	Wallac	SC/9200/21
Bovine serum albumin	Sigma	A4503
Ethanol Absolute	Sigma	E7023
Ethylendiaminetetra-acetic acid disodium salt (EDTA)	BDH	100935V
Golgi Plug	BD-Pharmingen	555029
Metrizamide	Sigma	69753
Paraformaldehyde	Sigma	P6148
PBS 10X	Invitrogen	14200-083
Polyoxyethylenesorbitan monolaurate (Tween-20)	Sigma	P1279
Saponin	Sigma	S7900
Sodium Azide	Sigma	S5886
Sodium Bicarbonate	Sigma	S6014
Sodium Chloride	Sigma	S5886
Sodium phosphate, dibasic anhydrous	Sigma	S7907

Reagent	Supplier	Catalogue Number
Sodium phosphate, monobasic anhydrous	Sigma	S8282
Sterile Water	Baxter/ 3S healthcare	TRF7114

Table 2.2: Table of reagents

2.1.3. Tissue Culture reagents

Name	Supplier	Catalogue Number
2 beta mecaptoethanol 500mM	Invitrogen	31350-010
Alkaline phosphatase substrate kit	Bio Rad	172-1063
Antibiotic/Antimycotic solution (Pen/ Strep)	Invitrogen	15240-062
Chicken egg albumin (OVA)	Sigma	A7641
Chicken gamma globulin (CGG)	Stratatech Scientific	003 000 002
Collagenase Type III	Lorne Laboratories	LS004183
Crystal Violet	Sigma	C3886
Deoxyribonuclease I (DNase I)	Sigma	D25
DNP-ficoll	Biosearch Technologies	F-1200-10

Name	Supplier	Catalogue Number
Dynabeads M459 Sheep anti-mouse IgG	Dynal UK	110.02
Dynabeads M459 Sheep anti-rat IgG	Dynal UK	110.08
Fetal Calf Serum (FCS) Lot 40Q1282F	Invitrogen	10106-169
Formalin	BDH	352276Y
Gentamycin	Invitrogen	15750-045
Guinea Pig Complement	VH Bio	CL4051
HBSS (Hank's Buffered Salt solution)	Invitrogen	14085-049
Heparin	LEO	505
Hepes Buffer 1M	Invitrogen	1530-056
Histopaque 1.083	Sigma	1.083-1
Horse Serum	Invitrogen	16050.098
Hydrocloric Acid HCl	BDH	190665R
Iscoves Modified Medium	Invitrogen	31980-022
Low endotoxin FCS Lot	Harlan	S-0001E
Modified Earles medium	Invitrogen	10370-021
NCTC	Invvitrogen	41350-026
Neomycin Sulphate	Sigma	N1876
Nycoprep 1.077A	Invitrogen	1002380N
OPD tablets	Sigma	P9187
Polyinosinic-polycytidylic acid (Polyl:C)	Sigma	P1530

Name	Supplier	Catalogue Number
Polymyxin 'B' sulphate	Sigma	P1004
RPMI 1640 medium	Invitrogen	21870076

Table 2.3: Table of tissue culture reagents

2.1.4. Solutions

2.1.4.1. Red cell lysis buffer

PBS with 0.83% ammonium chloride

2.1.4.2. RP10

RPMI with 10% FCS, 5% NCTC media, 1% Penstrep, 1% Hepes, 0.5%. gentamycin, 1% 2βME solution.

2.1.4.3. FACS buffer

HBSS solution, 2% horse serum, 0.1% sodium azide.

2.1.4.4. RP2.5

RPMI with 2.5% FCS, 5% NCTC media, 1% penstrep, 1% 2βME solution, 0.5% gentamycin.

2.1.4.5. Carbonate coating buffer

0.05M Na_2CO_3, 0.05M $NaHCO_3$, pH9.6

2.1.5. Plastics and Miscellaneous

Product	Company	Catalogue number
40μm cell strainers	Fahrenheit	352340
70μm cell strainers	Fahrenheit	352350
6 well plates	SLS	152795
24 well plates	SLS	143982
48 well plates	SLS	150687
96 well 'U' bottomed plates	SLS	163320
ELISA plates	SLS	168055A
ELISPOT plates	Millipore	MAHAS4510
FACS tubes	Fahrenheit	352008
Glass slides	Sigma	S8400
MoFlo tubes	Fahrenheit	352054
Petri dishes	SLS	101VR20

Table 2.4: Table of plastics and miscellaneous

2.1.6. Radioactive isotopes

Tritiated [3H] thymidine was purchased from Amersham Pharmacia Catalogue Number TRA120.

2.1.7. Recombinant cytokines

Cytokine	Company	Catalogue Number
Murine recombinant IL-2	R&D systems Europe	402-ML-020
Murine recombinant GM-CSF	R&D systems Europe	415-ML-005

Table 2.5: Table of recombinant cytokines used

2.1.8. Peptides

All the peptides were purchased from Mr Laurence Hunt of the IAH, Compton.

Name	Sequence	Description
2C peptide	SIYRYYGL	CD8 2C Transgenic TCR peptide.
GP1-1	KAVYNFATC	Dominant H2-K^b restricted epitope in LCMV.
GP1-3	SGVENPGGYCL	Sub Dominant (3^{rd}) H2-K^b restricted epitope in LCMV
GP2-1	SGEGWPYIACRTSIVGRAWE	Dominant I-A^b restricted epitope in LCMV Armstrong.
NP1-2	FQPQNGQFI	Sub Dominant (2^{nd}) H2-K^b restricted epitope in LCMV
NP2-2	GLKGPDIYKGVYQFKSVEFD	Sub Dominant (2^{nd}) I-Ab restricted epitope in LCMV Armstrong.

Name	Sequence	Description
NP2-3	GLKGPDIYKGVYQFKSVEFD	Sub Dominant (3^{nd})I-A^b restricted epitope in LCMV.
OVA	SIINFEKL	Dominant H2-K^b restricted epitope in ova. OT2 specific

Table 2.6: Table of peptides used

2.1.9. Antibodies, cell sorting, and FACS reagents

Antibody	Clone	Isotype	Supplier	Conjugate/ Format	Catalogue Number
Anti-Mouse CD2	RM2-5	Rat IgG_{2b}	BD-Pharmingen	Biotin	553110
Anti-mouse CD3e	145-2C11	ArmH IgG_1	BD-Pharmingen	Purified NA/LE R-PE	553057 553064
Anti-mouse CD4	GK1.5	Rat IgG_{2b}	BD-Pharmingen EJIVR EJIVR EJIVR BD-Pharmingen	Biotin Cy5 FITC Supernatant R-PE	553728 - - - 553730
Anti-Mouse CD5	57-7.3	Rat IgG_{2a}	BD-Pharmingen	R-PE	553022

Antibody	Clone	Isotype	Supplier	Conjugate/ Format	Catalogue Number
Anti mouse CD8a	YTS.169	Rat IgG_{2b}	EJIVR	Cy-5	-
		Rat IgG_{2b}	EJIVR	Supernatant	-
Anti Mouse CD11a	2D7	Rat IgG_{2a}	BD-Pharmingen	Biotin	01202A
Anti-Mouse CD11b	M1/70	Rat IgG_{2b}	BD-Pharmingen	Biotin	553309
			BD-Pharmingen	R-PE	553311
			EJIVR	Supernatant	-
Anti-Mouse CD11c	HL3	ArmH	BD-Pharmingen	Biotin	553800
		IgG_1	BD-Pharmingen	FITC	553801
			BD-Pharmingen	R-PE	553802
Anti-mouse Fc Block (CD16/32)	2.4G2	Rat IgG_{2b}	BD-Pharmingen	Purified	553142
Anti–mouse CD18	C71/16	Rat IgG_{2a}	BD-Pharmingen	R-PE	553293
Anti-mouse CD21	7G6	Rat IgG_{2b}	BD-Pharmingen	FITC	553818
Anti-mouse CD23	B3B4	Rat IgG_{2a}	BD-Pharmingen	R-PE	553139
Anti-Mouse CD24	M1/69	Rat IgG_{2b}	BD-Pharmingen	Biotin	01572D
	M1/69	Rat IgG_{2b}	BD-Pharmingen	R-PE	553262

Antibody	Clone	Isotype	Supplier	Conjugate/ Format	Catalogue Number
Anti-mouse CD25	PC61	Rat IgG_1	BD-Pharmingen	R-PE	09985B
Anti-mouse CD28	37.51	SyrH IgG_1	BD-Pharmingen	Purified NA/LE	553294
Anti-Mouse CD31	MEC 13.3	Rat IgG_{2a}	BD-Pharmingen	R-PE	553373
Anti-mouse CD40	3/23	Rat IgG_{2a}	BD-Pharmingen	Biotin	553789
Anti-mouse CD43	S7	Rat IgG_{2a}	BD-Pharmingen	R-PE	553271
Anti-mouse CD44	IM7	Rat IgG_{2b}	BD-Pharmingen BD-Pharmingen	Biotin CyChrome	553132 553135
Anti-mouse CD45.1 (Ly5.2)	A20	Mouse IgG_{2a}	EJIVR BD-Pharmingen	Biotin R-PE	- 553776
Anti-mouse CD45.2 (Ly5.1)	104	Mouse IgG_{2a}	BD-Pharmingen BD-Pharmingen	Biotin FITC	553771 553772
Anti-mouse CD45R (B220)	RA3-6B2	Rat IgG_{2a}	BD-Pharmingen BD-Pharmingen Caltag	Biotin CyChrome Cy-5	553086 553091 RM2611

Antibody	Clone	Isotype	Supplier	Conjugate/ Format	Catalogue Number
Anti-mouse CD49b	DX5	Rat IgM	BD-Pharmingen	R-PE	553858
Anti-mouse CD54	3E2	ArmH IgG_1	BD-Pharmingen	Biotin	553249
Anti-mouse CD80	16-10A1	ArmH IgG_2	BD-Pharmingen	Biotin	553767
Anti-mouse CD86	GL1	Rat IgG_{2a}	BD-Pharmingen	Biotin	09272D
Anti-mouse CD102	3C4 (mIC2/4)	Rat IgG_{2a}	BD-Pharmingen	Biotin	557441
Anti-mouse CD117	2B8 ACK45	Rat IgG_{2b} Rat IgG_{2b}	BD-Pharmingen BD-Pharmingen	APC R-PE	553356 553869
Anti-mouse CD127	B12-1	Rat IgG_{2b}	BD-Pharmingen	Biotin	555288
Anti-mouse CD138	281-2	Rat IgG_{2a}	BD-Pharmingen	Biotin	09342D
Anti-mouse CD162	2PH1	Rat IgG_1	BD-Pharmingen	R-PE	555306
Anti-mouse CD229.1	30C7	Rat IgG_{2b}	BD-Pharmingen BD-Pharmingen	Biotin FITC	557363 553117

Antibody	Clone	Isotype	Supplier	Conjugate/ Format	Catalogue Number
Anti-mouse IFN-γ	R4-6A2 XMG1.2 XMG1.2	Rat IgG_1 Rat IgG_1 Rat IgG_1	BD-Pharmingen BD-Pharmingen BD-Pharmingen	Purified Biotin R-PE	551216 554410 18115A
Anti-mouse IgM^{pan}	Polyclonal	goat	Southern biotech	R-RE	1021-09
Anti Mouse IgD^{pan}	11-26c.2a	Rat IgG_{2a}	BD-Pharmingen	FITC	02214D
Anti Mouse IgM^a	DS-1	Mouse IgG_1	BD-Pharmingen	Biotin	05092D
Anti Mouse IgM^b	AF6-78	Mouse IgG_1	BD-Pharmingen	Biotin	05102D
Anti Mouse $IgG_1{}^a$	10.9	Mouse IgG_{2a}	BD-Pharmingen	Biotin	05002D
Anti Mouse $IgG_1{}^b$	B68-2	Mouse IgM	BD-Pharmingen	Biotin	05172D
Anti Mouse $IgG_{2a}{}^a$	8.3	Mouse $IgG_{2a}{}^b$	BD-Pharmingen	Biotin	05022D
Anti Mouse $IgG_{2a}{}^b$	5.7	Mouse IgG_3	BD-Pharmingen	Biotin	05032D
Anti-mouse IL-4	11B11	Rat IgG_1	BD-Pharmingen	R-PE	554435

Antibody	Clone	Isotype	Supplier	Conjugate/ Format	Catalogue Number
Anti-mouse LPAM-1	DATK32	Rat IgG_{2a}	BD-Pharmingen	R-PE	553811
Anti-mouse Class II	TIB-120	-	EJIVR	Supernatant	-
Anti-mouse MADCAM-1	MECA-89	Rat IgG_{2a}	BD-Pharmingen	Biotin	553808
Anti-mouse TER119	TER-119	Rat IgG_{2a}	EJIVR	Supernatant	-
Anti-mouse Thy 1.2	J1J.10	Rat IgM	EJIVR	Supernatant	-
Streptavidin		-	Invitrogen BD-Pharmingen BD-Pharmingen BD-Pharmingen	RED670 PerCP Alkaline phosphatase Horse Raddish Peroxidase	19543-024 1303PA 13043E 13047E
TCRvβ panel	Various	Various	BD-Pharmingen	FITC	557004

Table 2.7: Table of antibodies used.

2.1.9.1. Tetramers

Tetramer	Peptide	Conjugate	Restriction	Company
LCMV-GP	KAVYNFATC	R-PE	H2-D^b	ProImmune
OVA	SIINFEKL	R-PE	H2-K^b	ProImmune
LCMV-NP	FQPQNGQF	R-PE	H2-D^b	ProImmune

Table 2.8: Table of tetramers used.

2.1.9.2. *In vitro* fluorescent reagents

Name	Size/MW	Company	Calalogue Number
Fluoresbrite®YG Microspheres	0.5μm	Polysciences Europe GmbH	17152-10
Alexa Fluor 647 Dextran	10000	Molecular probes	D1825
Alexa Fluor 488 Hydrazide	975	Molecular probes	A-10436
Dextran- fluorescein	40000	Molecular probes	D1845

Name	Size/MW	Company	Calalogue Number
Hyaluronic Acid, bodipy FL conjugate	-	Molecular probes	H23270

Table 2.9: Table of miscellaneous fluorescent reagents

2.2.Methods

2.2.1. Mice

All the mice were bred under specific pathogen free conditions at the Institute for Animal Health, Compton, UK or supplied by Charles River UK. The culling of mice was carried out using either the scheduled 1 method of asphyxiation by a rising concentration of CO_2 or by terminal anaesthesia.

2.2.2. Chimeras & Irradiation

Mice were irradiated using a Nordion Gammacell 40 irradiator (GC40 exactor, MDS Nordion International Inc. Kanata, Canada). The mice received varying amounts of irradiation depending on the protocol, as indicated in the results section. Mice were

given antibodies (polymixin B sulphate, 13mg/ml, and neomycin sulphate, 25mg/ml) for 8 weeks following Irradiation.

Cells for *in vitro* assays were irradiated for 3000 rads using a Nordion Gammacell 1000 irradiator (GC1000 elite, MDS Nordion International Inc. Kanata, Canada)

2.2.3. Thymectomies, and Sham Thymectomies

Mice were anaesthetised using hypoderm and ketamine, supplied by the staff of the small animals unit, at the Institute for Animal Health, Compton. Once suitably anaesthetised and immobilised, a small incision was made at the top of the sternum, and the thymus was removed by suction. The incision was then clamped together and the mice were allowed to recover. Sham thymectomies were carried out as normal thymectomies but without the removal of the thymus by suction. Mice were given antibiotics (polymixin B sulphate, 13mg/ml, and neomycin sulphate, 25mg/ml) for 5 weeks following the operation. The mice were left for 5 weeks to recover, before further manipulation occurred. Operations were performed by Dr David Tough.

2.2.4. Intrathymic injections

Mice were anaesthetised using hypoderm and ketamine. Once suitably anaesthetised and immobilised, a small incision was made in the top of the sternum, the thymus was then injected with approximately 20μl (10μl per lobe) of $B220^+$ depleted bone marrow. The incision was clamped together and the mice allowed left to recover. Mice were given antibiotics (polymixin B sulphate,

13mg/ml, and neomycin sulphate, 25mg/ml) for 5 weeks following the operation. Injections were performed by Dr David Tough.

2.2.5. Immunisations and infections

2.2.5.1. Ova Immunisation

Ova immunisations were carried out by the subcutaneous administration, of 100μg OVA and 100μg/ml polyI:C dissolved in 200μl of PBS. Mice which were immunised with Ova and polyI:C were then given two subsequent subcutaneous injections of 100μg/ml polyI:C (in 200μl of PBS), 24 and 48 hours after the primary inoculation. The spleen and LN were removed 8 days after the primary immunisation.

2.2.5.2. CGG Immunisations

Mice were pre-bled prior to immunisation to determine the titre and affinity of cross-reactive antibodies. CGG immunisations were carried out by the administration s.c. of 100μg CGG and 100μg/ml polyI:C dissolved in 200μl of PBS. Mice which were immunised with CGG and polyI:C were subsequently injected s.c. with polyI:C alone 24 and 48 hours after the primary immunisation. The serum was then collected 10 and 50 days later. Mice were then re-immunised with the same protocol of ova and polyI:C injections. The mice were then sacrificed 10 days after secondary immunisation, their blood and several organs were removed for *in vitro* analysis.

2.2.5.3. DNP Ficoll Immunisations

Mice were pre-bled prior to immunisation to determine their titre of cross-reactive antibodies. DNP-ficoll immunisations were carried out by the administration s.c. of 5μg DNP-ficoll in 200μl of PBS. The serum and spleen were then collected 10 days later.

2.2.5.4. LCMV Immunisations

Mice were infected i.p. with 2 $x10^5$ pfu of LCMV-Armstrong. The mice were sacrificed at various time points later, and various organs removed. The mice were housed under Category 3 conditions and all further manipulations of tissues were also carried out under Category 3 conditions, until rendered safe through viral inactivation.

2.2.6. Tissues

The tissues collected can be divided into three categories primary, secondary and tertiary lymphoid organs. The media in which the organs were collected, was determined by the fate of the organs. If the organs were to be used for FACS analysis then they were collected in PBS 2%FCS, if the organs were to be used for *in vitro* assays they were collected in sterile RPMI 2% FCS.

2.2.6.1. Primary Lymphoid organs

The thymus and bone marrow were surgically removed in an aseptic manner from dissected mice after confirmation of death.

2.2.6.2. Secondary Lymphoid tissues

Spleen, LN and peyer's patches, were aseptically removed from dissected mice after confirmation of death.

2.2.6.3. Tertiary lymphoid tissues,

Occasionally peritoneal cells, liver lymphocytes, blood lymphocytes and serum were also harvested.

2.2.6.3.1. Peritoneal cells

To obtain peritoneal cells the peritoneal cavity was injected with 5ml of ice cold RPMI supplemented with 5mM EDTA and 2%FCS media immediately upon confirmation of death. This was left for 2 minutes whilst gentle manipulation of the distended cavity was performed; the majority of media was then collected subsequently. A further injection of 5ml of ice-cold medium was given. This was again left for 2 minutes whilst gentle manipulation of the distended cavity was repeated; the remaining media was then harvested and pooled.

2.2.6.3.2. Liver lymphocytes

To obtain liver lymphocytes, 10ml of PBS supplemented with 2% FCS, 5mM EDTA and 0.01% heparin, was injected into the hepatic portal vein using a 25 gauge needle to perfuse the liver so as to remove any blood lymphocytes. The liver was continuously perfused until white in colour. The liver was then carefully removed so as to allow the gall bladder to be excised without rupture.

2.2.6.3.3. Blood Lymphocytes

To obtain blood lymphocytes, as soon after confirmation of death as possible, the mouse was dissected, renal veins severed, and the blood collected with a 1ml

syringe into 3ml PBS 0.01% Heparin. If peritoneal lymphocytes were also to be harvested from the same animal, then a thoracic cardiac puncture was performed to obtain the blood.

2.2.6.4. Serum

To obtain total serum, as soon after confirmation of death as possible, the mouse was dissected, renal veins severed, and the blood collected with a 1ml syringe into a plastic eppendorf without PBS or Heparin. If the animal was not to be culled, then a tail bleed was performed.

2.2.6.5. Tissue acquisition for LCMV titre determination

The spleen was surgically removed after death and a piece of tissue was taken and put in an eppendorf without media and then placed on dry ice. It was subsequently stored at -80°C until plaquing.

2.2.7. Tissue processing and cellular isolation

The cells were processed to a single cell suspension according to their fate and the organs that they originated from. The samples from the thymus, spleen and LN, if not isolating DC, were processed by using an abrasive method. The tissues were gently disrupted using two frosted glass slides (sterile glass slides if *in vitro* assays followed), thereby releasing the cells into media. If the cells were to be used for *in vitro* assays, they were re-suspended in RPMI supplemented with 2% FCS. Red

blood cells in the splenic cell samples were lysed using a hypo-osmotic, 0.83% ammonium chloride solution, red cell lysis buffer. If the cells were to be used for FACS (florescence activated cellular sorting) analysis, they were re-suspended in FACS buffer.

2.2.7.1. Bone marrow cells

Cells from the bone marrow were obtained by first cleaning the bones so that no muscle or sinew remains. Both ends of the bone were removed, and the contents flushed using a 25 gauge needle with either PBS, if the cells were to be used for FACS analysis, or sterile RPMI, if they were to be used for *in vitro* assays, both of which were supplemented with 2% FCS. If the cells were to be used to derive BMDCs then they were flushed with Iscoves modified medium supplemented with 10% LE FCS and Pen/Strep.

2.2.7.2. DC isolation

The organ from which the DCs were isolated was injected with a solution of Dnase1 and Collagenase IV. They were then placed in a rocking 37°C incubator for 30 minutes; 1ml of RPMI 2% FCS + 5mM EDTA was added for the last 5 minutes. The samples were pushed though 70μm filters, pelleted and re-suspended in 3ml of Nycodenz. This suspension was layered on top of 5ml of Nycodenz, with 1ml of LE-FCS placed on top of the sample. This was then spun at 2800rpm for 30minutes at 20°C (with the brake off). The enriched DCs were located at the interface of the Nycodenz and FCS.

2.2.7.3. Liver Lymphocyte isolation

The liver was dissected into small pieces using a razor blade before being pushed through a metal sieve, and re-suspended in RPMI 5% FCS. The cells were pelleted at 1200 rpm (4°C) for 7 minutes, then re-suspended in RPMI 5% FCS. The majority of hepatocytes were pelleted by centrifugation at 300rpm for 3 minutes at 4°C with the brake off. The supernatant, which contains the lymphocytes, was collected and spun down and the pellet re-suspended in digestion media (RPMI, 0.02% Collagenase IV, 0.002% Dnase1), where it was left at 37°C for 45 minutes with gentle agitation to digest the remaining tissue architecture and freeing the lymphocytes. The cells were then washed in RPMI before being re-suspended into a final volume of 1.6ml of RPMI, 2.4ml of 40% metrizamide in PBS. The cells were gently mixed, 1 ml of RPMI was overlaid and the cells were spun at 2600rpm 4°C for 25 minutes. Lymphocytes, found at the interface of the metrizamide solution and PBS, were then collected.

2.2.7.4. Blood Lymphocyte isolation

The blood from the mouse was collected in PBS supplemented with 0.01%heparin. It was the passed over an equal volume of histopaque 1088; the cells were then spun at 1600 rpm, 20°C for 30 minutes with the brake off. Lymphocytes, found at the interface of the histopaque and PBS, were then collected.

2.2.7.5. Plaque assays to determine the titre of LCMV

The tissue after being stored at –80°C was brought to room temperature before a known weight of the sample was homogenised using an electronic homogeniser in

1ml of RPMI until a single cell suspension was achieved. Dilutions of the sample were then made, and an aliquot of the dilutions was gently placed on a 95% confluent layer of vero cells in a 6 well plate. The cells were then left for 1 hr at 37°C before a layer of 1% Agar, 5%FCS, 0.5% pen/strep solution, 1x MEM, was placed over the top. The plates were incubated for 6 days before being fixed with 1% formalin. The plates were developed with crystal violet, so as to determine the number of plaques present in each sample.

2.2.7.6. Counting Cells

Cells were counted using a haemocytometer. Remaining red blood cells and non-viable cells were discriminated using phase-contrast whilst being counted.

2.2.8. Cell sorting and purification

2.2.8.1. Dynal Purification

Cells at a concentration of 2 x 10^7 cells/ml in RPMI 2%FCS were incubated with the appropriate antibody concentration for 20 minutes at 4°C. During this time, dynal beads were washed three times with RPMI 2%FCS to remove the preservative using a strong magnet, which allows the removal of the supernatant without disturbing the beads. The antibody labelled cells were washed to remove the excess antibody, and re-suspended with the appropriate amount of washed dynal beads (150μl of each dynal species per 1x10^8 total cells). The sample was gently agitated at 4°C for 45 minutes, before being placed on the magnet leaving

non-labelled cells in the supernatant. The beads were then re-suspended in fresh media before being replaced on the magnet, allowing for maximal yield of non-labelled cells.

2.2.8.2. Cell Purification by complement-mediated killing

Cells were incubated with the appropriate antibody concentration at $2x10^7$ cells/ml 2.5% FCS RPMI with 10% guinea pig complement for 45 minutes at 37°C. The cells were then filtered to remove dead cell clumps and washed to remove excess antibody and complement.

2.2.8.3. Cell Sorting on the MoFlo

Fluorescently-labelled cells were purified using a MoFlo sorter; cells were labelled with florescent antibody as described in section 2.2.10. Although a higher concentration of antibody was frequently needed due to a reduced sensitivity of the Moflo (compared to the FACS Calibur which was used for analysis), as well as the greater staining volumes used. The cells were also stained in PBS 2%FCS, to allow their further use in functional assays. Immediately before sorting they were filtered through a 40μm filter to prevent blockage of the MoFlo. The cells were sorted into 0.5ml of FCS to increase cell viability. Either Mr Andrew Worth or Miss Janine Oldham operated the MoFlo.

2.2.8.4. Purification for Bone marrow For Chimeras

Bone marrow cells to be injected into an irradiated mouse were manipulated to remove a variety of cell types, the most common of which was the $CD3^+$ subset.

This was done mainly by complement-killing, using an antibody against Thy 1.2. Occasionally B cells were also removed. This was done by dynal bead depletion using anti-B220 and anti-mouse IgG; the latter has cross reactivity to murine IgM and IgD thereby removing the majority of the B cells. Where indicated, all lineage positive cells were removed this was done by using an antibody cocktail and dynal bead negative selection. More details of this method can be found in the text. Purified cells were washed three times with PBS to remove residual FCS before being filtered through a 40μm filter in preparation for *i.v.* injection.

2.2.9. Cell Culture

2.2.9.1. Bone Marrow derived Dendritic cells

A single cell suspension of bone marrow cells was re-suspended in Iscoves Modified Medium supplemented with 10% LE FCS, 1% Pen strep, 5 mM 2βME, and 10μg/ml GMCSF. The medium was changed 3 days after culture set up, and the cells harvested 8 days after initial culture.

2.2.10. Immunoflourescent staining

2.2.10.1. Surface FACS Staining

Cells for FACS staining were aliquoted out into 5ml polypropylene tubes with 1 $x10^6$ cells per tube; the cells were then washed with FACS. Fc sites were blocked by the addition of Fc Block (anti-CD16/CD32) for 15 minutes whilst the cells were kept at 4°C. Antibodies were added at an appropriate dilution for 20 minutes at 4°C in the dark to prevent photobleaching. The sample was then washed with 2ml of FACS buffer to remove excess antibody. If any of the antibodies were biotinylated, binding was visualised by adding flurochrome-conjugated streptavidin, and the samples were incubated for 15 minutes at 4°C in the dark. The sample was then washed with 2ml of FACS buffer to remove excess antibody. If stained samples were not to be analysed by FACS within 12hrs the cells were fixed with 0.5ml PBS and 2%PFA, and left overnight at 4°C in the dark.

2.2.10.2. Tetramer Staining

Cells to be tetramer stained were depleted of B cells and Fc^+ cells, usually by dynal depletion $B220^+$ and $CD16/CD32^+$ cells. The cells to be stained were then incubated with an appropriate quantity of tetramer in wells of a 96-well u bottomed plates, for 45 minutes at room temperature in the dark. Samples were then washed before further staining of cell surface markers.

2.2.10.3. Intracellular staining

Cells were stimulated with the appropriate peptide or protein for 8hrs in RP10 at 37°C, before golgi stop was added (2μl/ml) for an additional two hours with 10units per ml of IL-2. The cells were then harvested and washed in FACS buffer, before surface staining, as described previously (section 2.2.10). After washing, the cells were fixed using 4%PFA/PBS, and then left for a minimum of 2hrs. Samples were then washed twice in permeabilisation buffer (PBS with 0.1% saponin, 0.1% sodium azide, 1% FCS). Anti-cytokine antibodies were then added for one hour at 4°C in the dark. Cells were then washed with FACS buffer and analysed on the FACS.

2.2.10.4. Flow cytometry and analysis

For examining cell surface phenotype, flow cytometry was carried out using a FACScalibur, and the data was analysed using mainly Cell Quest (BD). Summit (cytomation) was used for analysis of MoFlo acquired samples.

2.2.10.5. Statistical analysis

Analysis of results was perfomed using Microsoft Excel 2002 software, where two-sample Student's t-tests were performed. Student's t-tests were used to provide an exact test of the equality of normally distributed means from two independent sample populations. Assumptions made: 1, the samples were drawn from normally distributed populations, 2, the population difference was less than 2, and 3, there were no outliers.

2.2.11. *Ex vivo* Assays

2.2.11.1. Elispots

There were two types of Elispots used in this thesis, IFN-γ Elispots, and B cells specific Elispots. They differ in the setting up and the biotinylated detection antibodies used, but after that the procedure is identical.

2.2.11.1.1. IFNγ Elispots.

Elispot plates were coated with purified anti-IFN-γ antibody in 0.05M carbonate coating buffer (pH9.6) overnight at 4°C. The plate was washed 3 times with filtered PBS, and blocked for at least 1hr at 37°C with RP10. Stimulator cells, which were CD8 depleted splenocytes (negative depletion by dynal beads see section 2.2.8.1), were added a $5x10^5$ cells/well. A variety of effector cell concentrations were added to each well; usually between $5x10^3$ and $5x10^5$. The cells were stimulated with a known concentration of peptides and/or protein. The cells were then left at 37°C for between 12 to 36hrs, depending on stimulation, before development.

2.2.11.1.2. B cell specific Elispot

CGG or DNP-ficoll was coated at 10μg/ml in carbonate coating buffer (pH9.6) overnight at 4°C. This was then removed and the plate washed 3 times with PBS. The plate was then blocked for 1 hour at 37°C using RP10. To focus on antibody production by plasma cells, sIg^+ B cells were depleted using sheep anti-mouse dynal beads, before being plated out at various concentrations. The plates were left at 37°C and 5% CO_2 for 12 hours before development.

2.2.11.1.3. Development of ELISPOT Plates

After the incubation period described above, media and cells were discarded. The plates were washed six times with PBS and five times with PBS-0.05%Tween20 on both sides of the membrane. The biotinylated detection antibody, specific for either IFNγ or a particular Immunoglobulin isotype, was then added in a suitable dilution of PBS-0.05%Tween20 and left for 4 hrs at room temperature. Subsequently, the antibody was discarded and the plate blotted. The plate was then washed five times with PBS-0.05%Tween-20 on both sides of the membrane, and an anti-biotin alkaline phosphatase antibody was added in a suitable dilution of PBS-0.05%Tween20 for 4hr at room temperature. The plate was then washed on both sides of the membrane, 3 times with PBS-0.05%Tween-20 and then 3 times with PBS. The plate was then developed using an alkaline phosphatase development kit, as described by the manufacturer. The development time was variable each time, and then the reaction was stopped using tap water. The ELISPOT plates were then counted using a Lecia stereo microscope.

2.2.11.2. Antigen Specific ELISAs

Antigen-specific antibodies found in the sera of immunised mice were measured by ELISA. The antigen was coated on a plastic plate (FACON 3912) at 5μg/ml in 0.05M Carbonate coating buffer (pH9.6) overnight at room temperature in a humidified box. The plates were then blocked using a solution of 4% milk powder in PBS and incubated for 45 minutes at 37°C. The plates were washed three times in wash buffer, PBS 0.05% tween-20. 100μl of diluted serum was added, dilutions were made in PBS 1% milk powder and 0.2% Tween-20. After one-hour incubation at room temperature in a humidified box, the plates were washed three

times in PBS-0.05% tween-20. 100μl of the secondary antibody was then added in PBS 0.1% Milk powder. After one-hour incubation at room temperature in a humidified box, the plates were washed three times in PBS-0.05% tween-20. 100μl of streptavidin - horseradish peroxidase, in PBS 1% milk powder, was added and the plate left for one hour at room temperature in a humidified box. The plates were washed three times in PBS-0.05% tween-20. The ELISA was developed using Sigma OPD tablets; the reaction was stopped by adding 50μl 3M HCl. The plates were read at 492nm, using a Spectramax 340, from molecular devices (Wokingham, UK) and analysed using Softmax Pro software.

2.2.12. *In vitro* functional Assays

The ability of the DCs to stimulate T cells can be measured by their ability to uptake, and to present antigen. The uptake ability of DCs was assayed using fluorescently labelled particles of different sizes, which are internalised by different uptake pathways; uptake of fluorescent particles was monitored by FACS. The ability of DCs to present antigen was assayed by loading the DCs with specific peptides and/or protein and measuring the proliferation of transgenic T cells by the incorporation of tritiated thymidine. Mixed leukocyte reactions (MLR's) were also used to assess the responsiveness of the T cells to allotypic stimulation and the responsiveness of the DCs to affect allotypic stimulation. T and B cells were non-specifically stimulated by cross-linking of either their cell surface molecules by immobilised antibodies.

2.2.12.1. Uptake Assays

BMDCs were assessed for their ability to internalise antigen. This was performed by measurements of phagocytosis of fluorescent microspheres (0.5μm in diameter), endocytosis of different sizes of fluorescent dextran beads (mw 10,000 & 40,000) and pinocytosis using a fluorescent dye (mw 975).

DCs were isolated using nycodenz (see Section 2.2.7.2.) and were re-suspended in RPMI-2%FCS, at a concentration of 1 x 10^7/ml. 100μl solutions of the particles to be used were added to DCs in a FACS tube. The tubes were kept on ice in the dark, until either transferred to a 37°C water bath, or left on ice for a negative control, for various periods of time. The uptake assay was then halted by the addition of 4ml of ice cold FACS solution. The cells were washed 3 times before proceeding to surface staining (see section 2.2.12.)

2.2.12.2. Non-specific *in vitro* proliferation assays

2.2.12.2.1. Mixed leukocyte reactions (MLRs)

$CD44^{+/+}$ and $CD44^{-/-}$ cells were assessed for their ability to act as either stimulators or responders in allogeneic MLRs with Balb/c cells. 10^5 responder cells in RP10 were added to wells of 96 well plates with $2x10^5$ - 5 $x10^4$ CD8 depleted irradiated (3000 Rads) splenocytes or 10^4 - 10^3 irradiated BMDCs.

2.2.12.2.2. Non-specific T cell stimulation assay

An optimal concentration of anti-CD3 (1μg/ml) and anti-CD28 (2.5μg/ml) in 100μl PBS was added to wells of 96 well U bottomed plates, and left overnight at 4°C.

The plate was washed 3 times with PBS, before 10^5 cells per well of T cells were added.

2.2.12.2.3. Non-specific B cell stimulation assay

An optimal concentration of anti-IgM (5μg/ml) in 100μl PBS was added to wells of a 96 well U bottomed plates, and left overnight at 4°C. The plate was washed 3 times with PBS, before 10^5 B cells were added for non-specific stimulations.

2.2.12.3. Antigen specific T cell assays

Either BMDCs or CD8-depleted splenocytes were incubated with a range of concentrations of protein or peptide at 37°C in RP10 for 2-4 hrs. The cells were then irradiated (3000 rads) and washed three times to remove the free protein or peptide. For splenocyte 10^5 stimulators were then added to wells of a 96 well U bottomed plate. Different numbers of BMDCs were tested for stimulation of antigen specific transgenic T cells. The effector T cells were MHCII depleted.

2.2.12.4. Proliferation assays

All proliferation assays were set up in 96 well u bottomed plates. The cells were stimulated by various methods (see section 2.2.12.2 – 2.2.12.3).

At various times after the start of the culture 1μCi. of tritiated thymidine was added per well. This was left for 16hrs after which the plate was placed at –20°C to stop further proliferation. The plates from the proliferation assays were thawed to room temperature before being harvested. The solid contents of the wells were transferred on to a filtermat, which was then washed 5 times with distilled water to remove the non-incorporated thymidine. The filtermats were left to dry, then

saturated with beta scintillant and sealed in plastic bags. The tritiated thymidine present on the filter was then measured by a Wallac 1450 microbeta trilux (PerkinElmer, Boston, USA).

3. Chapter 3: Fitness of the $CD44^{-/-}$ lymphocytes when placed in direct competition with $CD44^{+/+}$ lymphocytes in a steady state.

3.1. Introduction

CD44, a widely expressed transmembrane glycoprotein, has had many functions ascribed to it (see Section 1.3.). Most of the studies which have attributed some functional relevance to CD44 have been accomplished by using monoclonal antibodies which interfere with the ability of CD44 to bind to its various ligands. CD44-dependent processes have been shown to include lymphocyte activation and migration, adhesion, and haematopoiesis. Surprisingly, when $CD44^{-/-}$ mice were generated, there were only a few minor defects in the mature adult. These included (1) an altered tissue distribution of myeloid progenitors, (2) an increase in the size of granulomas in response to *Cryptosporidium parvum* infection, and (3) increased tumorigenicity of tumours derived from transformed $CD44^{-/-}$ fibroblasts, (219). In this study, T and B cell development were found to be normal, as were T and B cell functional responses to VSV or LCMV infection, or ovalbumin immunisation. Another group also found normal T and B cell development, although they did observe that mature $CD44^{-/-}$ T cells appeared to have a slight deficiency in entering the adult thymus; this was observed whether the recipient thymus was $CD44^{-/-}$ or $CD44^{+/+}$(220).

Further studies using these mice, and other transgenics which have deletions in certain various exons, have provided additional information on the functional importance of CD44, which will be covered in greater detail in Section 6.

However when examining the literature as a whole, there appears to be a discrepancy in the functional evidence for the importance of CD44 in lymphocyte development. Antibody blocking studies in $CD44^{+/+}$ mice show essential roles for CD44 in T cell development and bone marrow homing, but $CD44^{-/-}$ mice have subtle deviancies in lymphocyte re-circulation and myeloid progenitor distribution.

A functional role for CD44 in T cell development has been demonstrated by several groups each using different assays. Anti-CD44 antibodies prevented foetal liver cells, which are comparable to adult bone marrow cells, from entering the foetal thymus in thymic organ cultures (221). Anti-CD44 antibodies prevented rosette formation between thymocytes and stromal cells and therefore T cell development in *in vitro* cultures (222), and again anti-CD44 antibodies reduced T cell development in Foetal Thymic Organ Cultures. (128).

A functional role for CD44 in bone marrow homing has been shown in studies where co-injection of anti-CD44 antibodies with bone marrow cells, inhibited entry of the bone marrow cells to the bone marrow, (223). Anti-CD44 antibodies injected for 3 days before injection of bone marrow cells, still inhibited the bone marrow cells from seeding, (224). In addition anti-CD44v10 antibodies have been shown to prevent homing of myeloma cells to bone marrow and to prevent the adhesion of the myeloma cells to the bone marrow epithelium. (225).

A role for CD44 in B cell development has been described by the blocking of lympho-haematopoiesis in long-term bone marrow cultures by anti-CD44 antibodies (226).

Why an important role for CD44 has been indicated by antibody blocking studies but not in $CD44^{-/-}$ mice is unclear. One possibility is that when CD44 is absent from early on in embryogenesis, the resulting deficit leads to the induction of other genes, which could replace CD44 function. As described in the introduction, CD44 binds to its ligand, mainly hyaluronate as part of the extracellular matrix, which is in turn bound to CD44 expressed by the epithelial or stromal cells. Since CD44 is not the only receptor for hyaluronate, in the $CD44^{-/-}$ mice other receptors could potentially bind hyaluronate, and together with other adhesion molecules, compensate for the lack of CD44 and therefore allow normal development and normal function of lymphocytes in the $CD44^{-/-}$ mice. However, it is also possible that blocking antibodies, in addition to disrupting interactions between CD44 and its ligand, cause unintended effects (e.g. cross-linking and signalling, targeting to phagocytes) that are unrelated to a physiological function for CD44.

Therefore, there are potential drawbacks to investigating the role of CD44 in lymphocyte development and function by comparing $CD44^{+/+}$ and $CD44^{-/-}$ mice, or by injecting large quantities of blocking antibodies. To avoid these problems, we chose to compare directly the behaviour of $CD44^{-/-}$ and $CD44^{+/+}$ cells in the same host. Mixed bone marrow chimeras were generated by the injection of $CD44^{+/+}$ and $CD44^{-/-}$ progenitors into an irradiated $CD44^{+/+}$ host. This allows $CD44^{-/-}$ and $CD44^{+/+}$ haematopoietic cells to develop in an environment where CD44 is present and bound naturally to its ligands. This system should reveal any advantage of

CD44 expression in lymphocyte development, in an environment where compensatory mechanisms are unlikely to be established, and without the potential problems associated with antibody injections.

3.2.AIM

To generate mixed bone marrow chimeras and to compare the fitness of $CD44^{+/+}$ and $CD44^{-/-}$ cells when they are placed in direct competition with each other. This was executed in both $CD44^{+/+}$ and $CD44^{-/-}$ hosts, so as to investigate any influence of the environment.

3.3.Results

3.3.1. Phenotypic description of Chimeras: $CD44^{+/+}$ ($Ly5.2^{+}$) and $CD44^{-/-}$ ($Ly5.1^{+}$) donors → $CD44^{+/+}$ ($Ly5.2^{+}$) hosts

Ly5.2 ($CD44^{+/+}$) and $CD44^{-/-}$ mice were sacrificed and their bone marrow T cell depleted by antibody mediated complement cytotoxicity. The cells were mixed in a 1:1 ratio and injected into irradiated host Ly5.2 mice (900 rads). Once immune reconstitution had occurred they were sacrificed, and their phenotype was analysed by FACS. Cells derived from the $CD44^{-/-}$ progenitor cells were identified using an anti-Ly5.1 (CD45.2) antibody.

These mice had normal cell numbers in spleens (see appendix 1), thymi and LN, and normal numbers and percentages of total T and B cells, (Figures 3.1A, 3.2A). However when the T and B cells from these chimeras were analysed there was a statistical difference in the origin of these cells (Figures 3.1B, 3.2B). It appeared that $CD44^{-/-}$ progenitors preferentially developed into B cells whereas the $CD44^{+/+}$ cells developed into T cells. This was more noticeable when the B:T cell ratio among the $CD44^{-/-}$ cells was calculated and compared to the total B:T cell ratio (Figures 3.1C, 3.2C). There were 6.2 $CD44^{-/-}$ B cells to every $CD44^{-/-}$ T cell in the spleen, but overall 1.9 B cell to every T cell in the spleen. In the LN there was a similar pattern with 2.8 $CD44^{-/-}$ B cells per T cell in the LN and overall 0.8 B cell to every T cell in the LN. After finding this discrepancy it was decided to further investigate the contribution of $CD44^{-/-}$ cells to T cell subsets and B cell subsets.

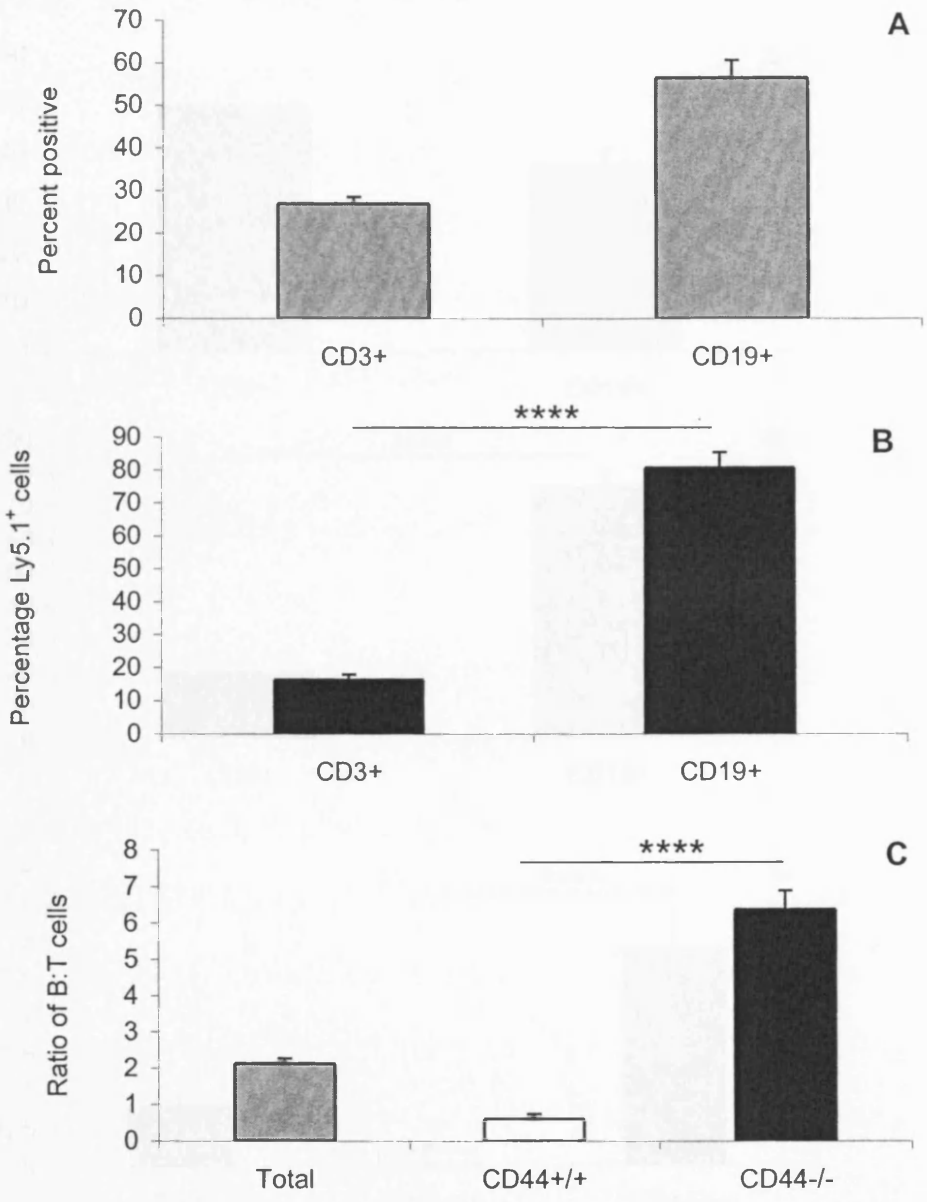

Figure 3.1. Analysis of T and B cells in spleens of $CD44^{+/+}$ & $CD44^{-/-}\rightarrow$ $CD44^{+/+}$ chimeras.

A. Percentages of T and B cells in spleen. B. The percentage of $Ly5.1^{+}$ cells in each splenic population. C. The B:T cell ratio among total, $CD44^{-/-}$ and $CD44^{+/+}$ populations. The data are mean ± s.e.m. obtained from 3 independent groups of chimeras (3 mice per group), over an 8-month period from date of reconstitution. P values, 2-sample t test; ****, $P<0.001$

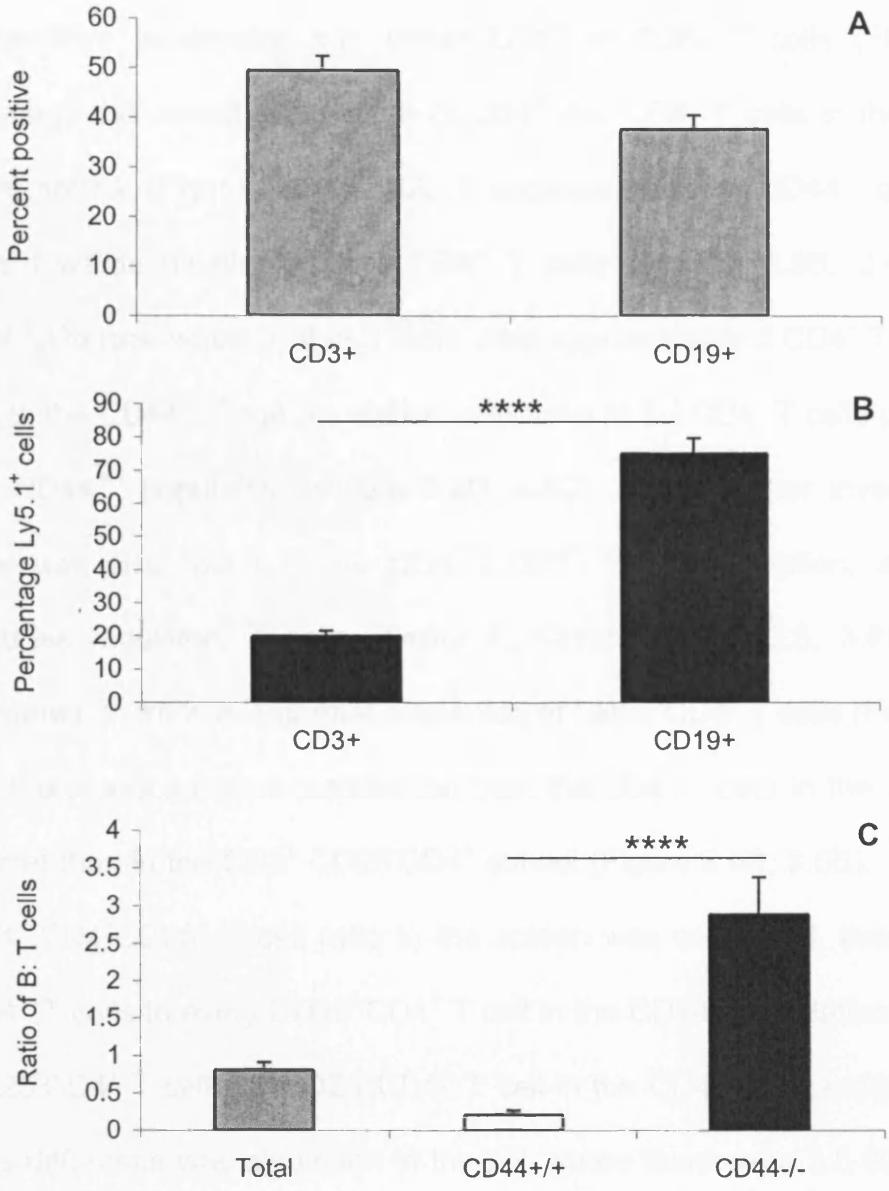

Figure 3.2. Analysis of T and B cells in lymph nodes of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A. Percentages of T and B cells in lymph nodes. B. The percentage of $Ly5.1^{+}$ cells in each population. C. The B:T cell ratio among total, $CD44^{-/-}$ and $CD44^{+/+}$ populations. The data are mean ± s.e.m. obtained from 3 independent groups of chimeras. (3 mice per group), over an 8-month period from date of reconstitution. P values, 2-sample t test; ****, $P<0.001$

In the spleen and LN, a difference was observed in the ability of $CD44^{-/-}$ progenitors to develop into either $CD4^+$ or $CD8^+$ T cells (Figures 3.3, 3.4). Although the overall percentage of $CD4^+$ and $CD8^+$ T cells in the spleen and LN were normal (Figures 3.3A, 3.4A), it appeared that the $CD44^{-/-}$ progenitors had a bias towards developing into $CD4^+$ T cells (Figures 3.3B, 3.4B). When the CD4:CD8 ratio was calculated there were approximately 2 $CD4^+$ T cells per $CD8^+$ T cell in the $CD44^{-/-}$ T cell population compared to 1.4 $CD4^+$ T cells per $CD8^+$ T cell in the $CD44^{+/+}$ population (Figure 3.3C, 3.4C). Upon further investigation another bias was also found in the $CD4^+$ $CD25^+$ T cell population, a population that includes regulatory T cells (Taylor P, 1311) (Figures 3.5, 3.6). In the mixed chimeras, there was a normal proportion of $CD25^+CD4^+$ T cells (Figure 3.5A, 3.6A), but there was a higher contribution from the $CD44^{-/-}$ cells in the $CD3^+CD4^+CD25^+$ subset than in the $CD3^+$ $CD25^-CD4^+$ subset (Figure 3.5B, 3.6B). When the $CD25^-$ $CD4^+$:$CD4^+CD25^+$ T cell ratio in the spleen was calculated, there were 6 $CD25^-$ $CD4^+$ T cells to every $CD25^+CD4^+$ T cell in the $CD44^{-/-}$ population, compared to 11 $CD25^-CD4^+$ T cell per $CD25^+CD4^+$ T cell in the $CD44^{+/+}$ population (Figure 3.5C). This difference was also seen in the LN, where there were 3.5 $CD25^-CD4^+$ T cells to every $CD25^+CD4^+$ T cell in the $CD44^{-/-}$ population, compared to 6.5 $CD25^-CD4^+$ T cell per $CD25^+CD4^+$ T cell in the $CD44^{+/+}$ population (Figure 3.6C).

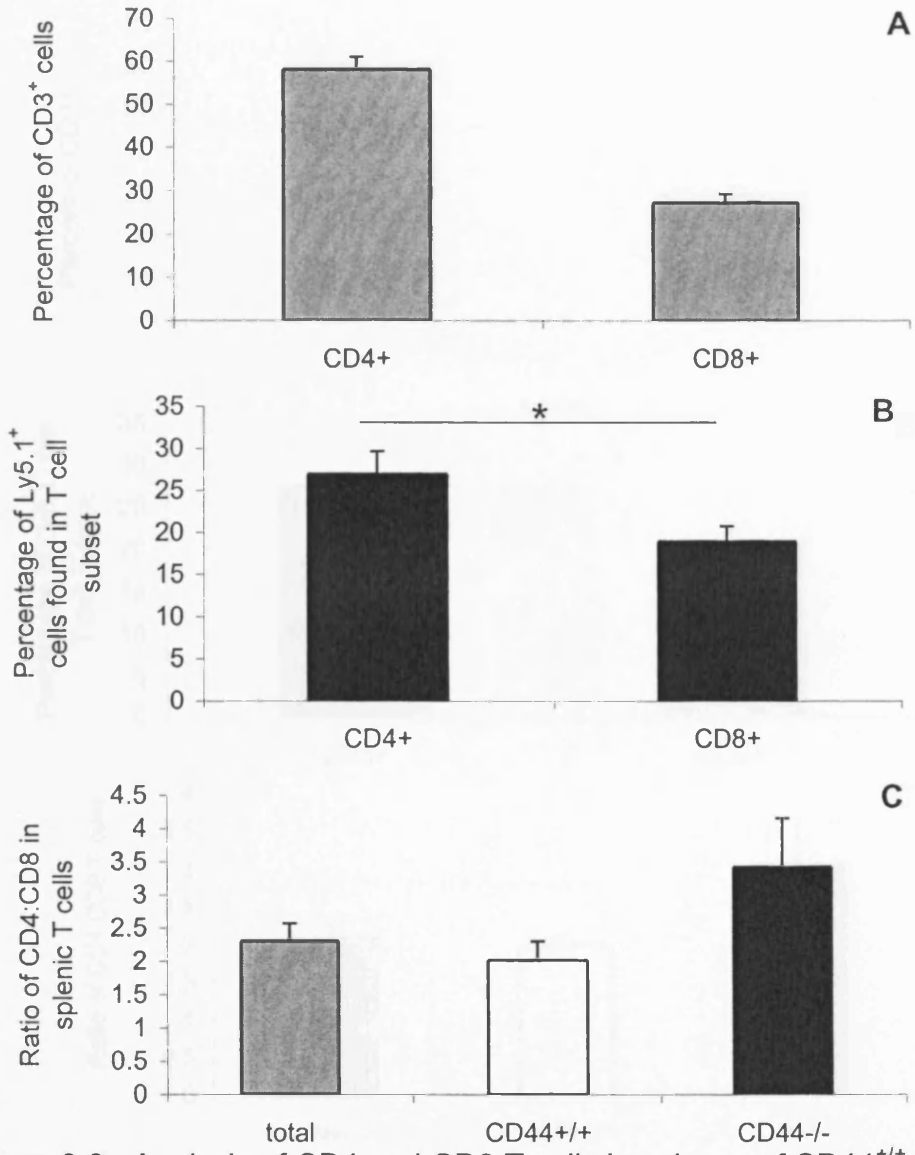

Figure 3.3. Analysis of CD4 and CD8 T cells in spleens of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A. Percentages of $CD4^+$ and $CD8^+$ $CD3^+$ cells in spleen. B. The percentage of $Ly5.1^+$ cells in each splenic population. C. The CD4:CD8 cell ratio among total, $CD44^{-/-}$ and $CD44^{+/+}$ populations. Data are mean ± s.e.m. obtained from 4 independent groups of chimeras (3 mice per group). P values, 2-sample t test; *, $P<0.05$

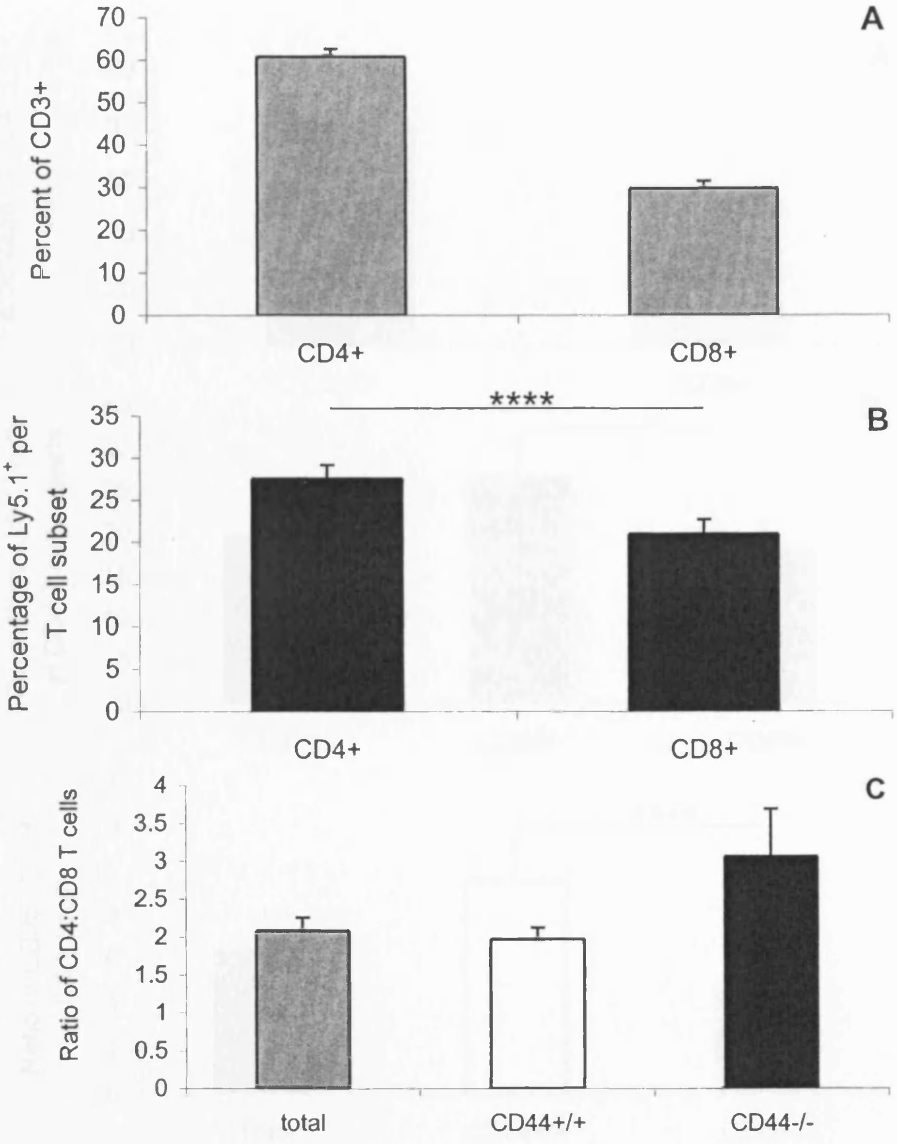

Figure 3.4. Analysis of CD4 and CD8 T cells in lymph nodes of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A. Percentages of $CD4^+$ and $CD8^+$ $CD3^+$ cells in lymph nodes. B. The percentage of $Ly5.1^+$ cells in each T cell subset. C. The CD4:CD8 cell ratio among total, $CD44^{-/-}$ and $CD44^{+/+}$ populations. Data are mean ± s.e.m. obtained from 2 independent groups of chimeras (3 mice per group). P values, 2-sample t test; ****, $P<0.001$

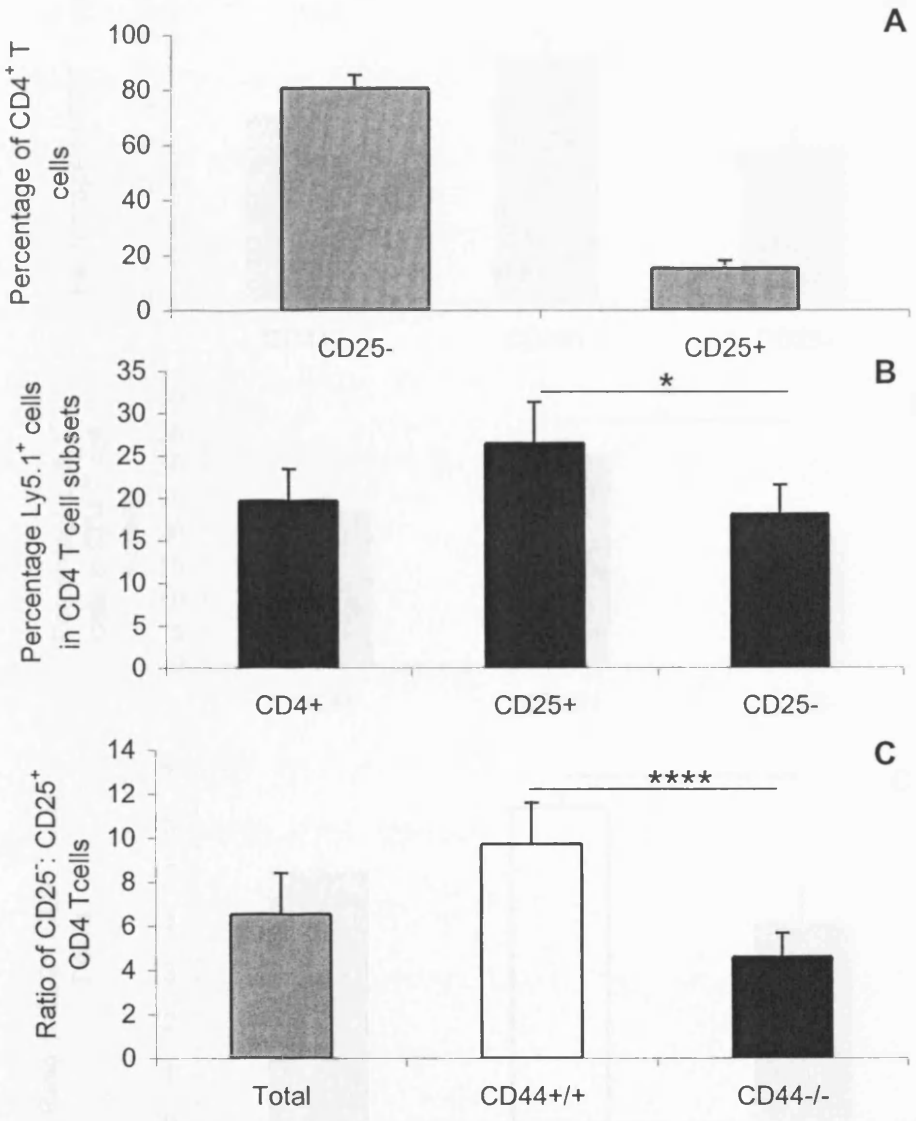

Figure 3.5. Analysis of CD4 subsets in spleens of $CD44^{+/+}$ & $CD44^{-/-}\rightarrow$ $CD44^{+/+}$ chimeras.

A. Percentages of $CD25^{+}$ and $CD25^{-}$ $CD4^{+}$ T cells. B. The percentage of $Ly5.1^{+}$ cells in each CD4 T cell subset. C. The $CD25^{-}$:$CD25^{+}$ $CD4^{+}$ T cell ratio among total, $CD44^{-/-}$ and $CD44^{+/+}$ populations. Data are mean ± s.e.m. obtained from 2 groups of 3 mice. P values, 2-sample t test; ****, $P<0.001$, *, $P<0.05$

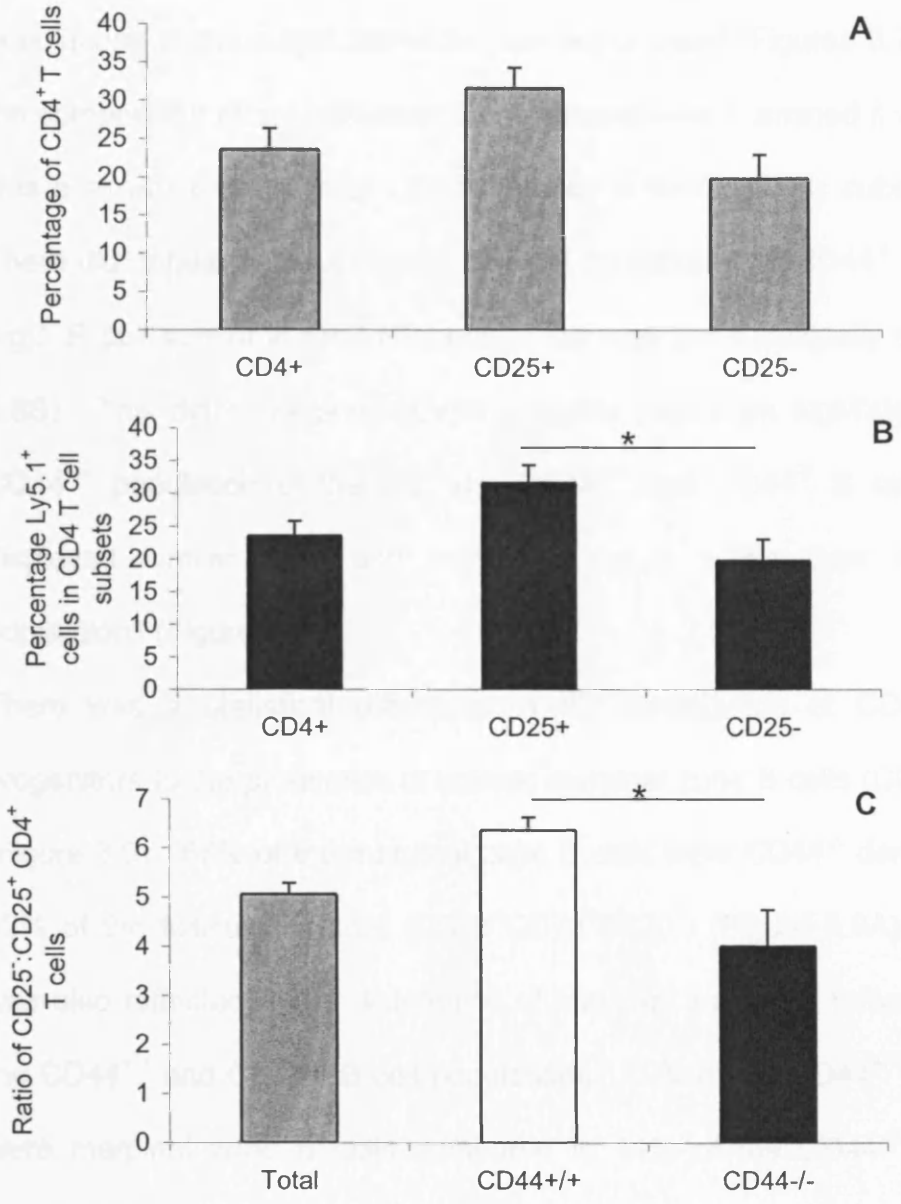

Figure 3.6. Analysis of CD4 subsets in lymph nodes of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A. Percentages of $CD25^+$ and $CD25^-$ $CD4^+$ T cells. B. The percentage of $Ly5.1^+$ cells in each CD4 subset. C. The $CD25^-$:$CD25^+$ $CD4^+$ T cell ratio among total, $CD44^{-/-}$ and $CD44^{+/+}$ populations. Data are mean ± s.e.m. obtained from 1 groups of 3 mice, and 1 group of 2 mice. P values, 2-sample t test; *, $P<0.05$

The distribution of sIgM$^+$ and sIgD$^+$ cells in the B220$^+$ splenic and lymph node populations of the mixed chimeras was not unusual (Figures 3.7A, 3.8A). When the composition of the individual B cell subsets was examined it was evident there was a similar proportion of CD44$^{-/-}$ B cells in each splenic subset (Figure 3.7B). There did appear to be a decrease in the contribution of CD44$^{-/-}$ cells to the sIgM$^-$ sIgD$^-$ B cell subset in the LN although this was not statistically significant (Figure 3.8B). This did corresponded with a higher proportion sIgM$^-$sIgD$^-$ B cells in the CD44$^{+/+}$ population of the LN. The CD44$^{+/+}$ and CD44$^{-/-}$ B cells of the spleen displayed similar sIgD and sIgM expression within their respective B220$^+$ populations (Figure 3.7C).

There was a statistical difference in the contribution of CD44$^{-/-}$ vs. CD44$^{+/+}$ progenitors to the production of splenic marginal zone B cells (CD21$^+$CD23$^-$B220$^+$) (Figure 3.9). 67% of the marginal zone B cells were CD44$^{-/-}$ derived compared to 52% of the follicular B cells (CD21$^+$CD23$^+$B220$^+$) (Figure 3.9A). This difference was also reflected in the distribution of marginal zone and follicular B cells within the CD44$^{+/+}$ and CD44$^{-/-}$ B cell populations. 10% of the CD44$^{+/+}$ B cell population were marginal zone B cells compared to 14% of the CD44$^{-/-}$ B cells (Figures 3.9B,C). This is highlighted, when the ratio of marginal zone B cells (CD21$^+$CD23$^-$ B220$^+$) and follicular B cells (CD21$^+$CD23$^+$B220$^+$) is calculated, (Figure 3.9D). Among CD44$^{-/-}$ B cells, there were 5 follicular B cells for every marginal zone B cell. In contrast, there were 8 folicular B cells for every marginal zone B cell among the CD44$^{+/+}$ cells.

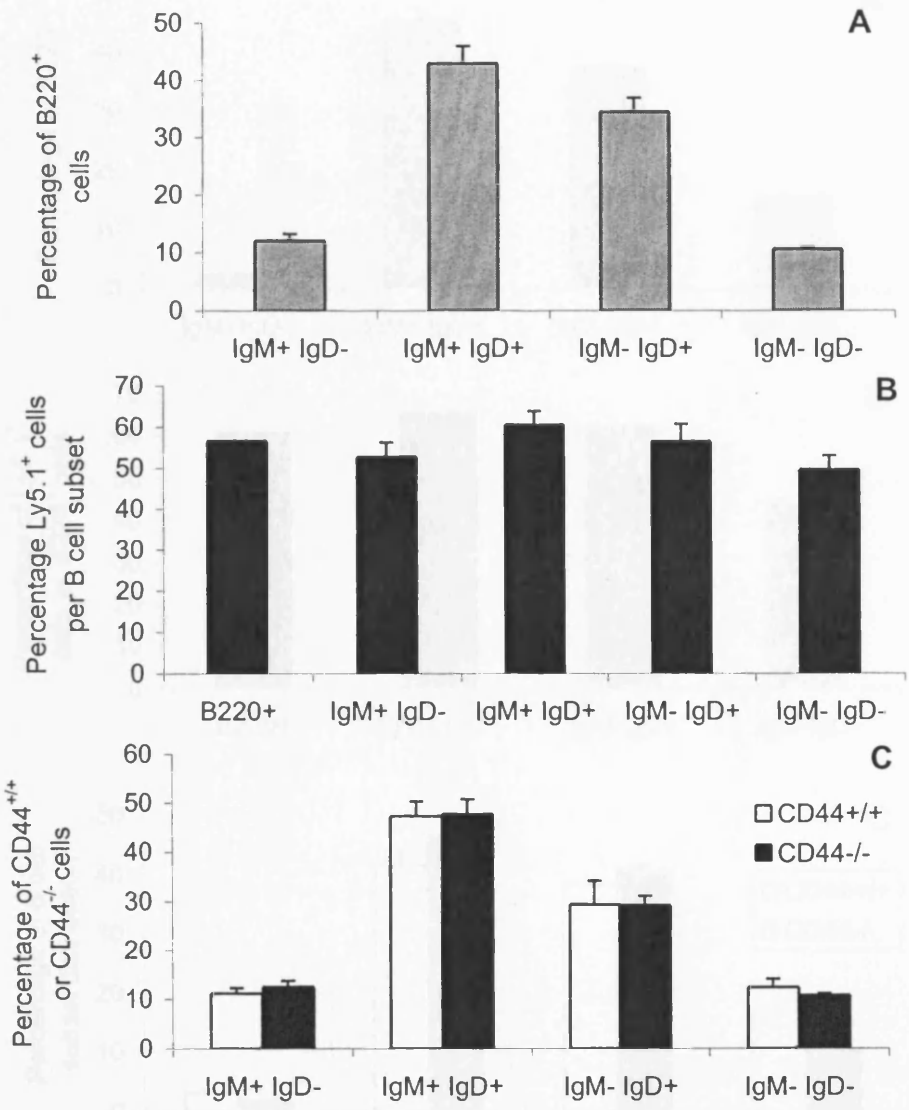

Figure 3.7. Distribution of sIgM$^+$ and sIgD$^+$ B cells in the spleens of CD44$^{+/+}$ & CD44$^{-/-}$→ CD44$^{+/+}$ chimeras.

A. Distribution of B cells subsets defined by sIgM and sIgD expression in splenic B220$^+$ cells. B. Contribution of CD44$^{-/-}$ cells to each subset of B220$^+$ cells as defined using sIgM and sIgD staining. C. Distribution of sIgM and sIgD staining among CD44$^{-/-}$ or CD44$^{+/+}$ B220$^+$ cells. Data are mean ± s.e.m. obtained from 1 group of 3 mice.

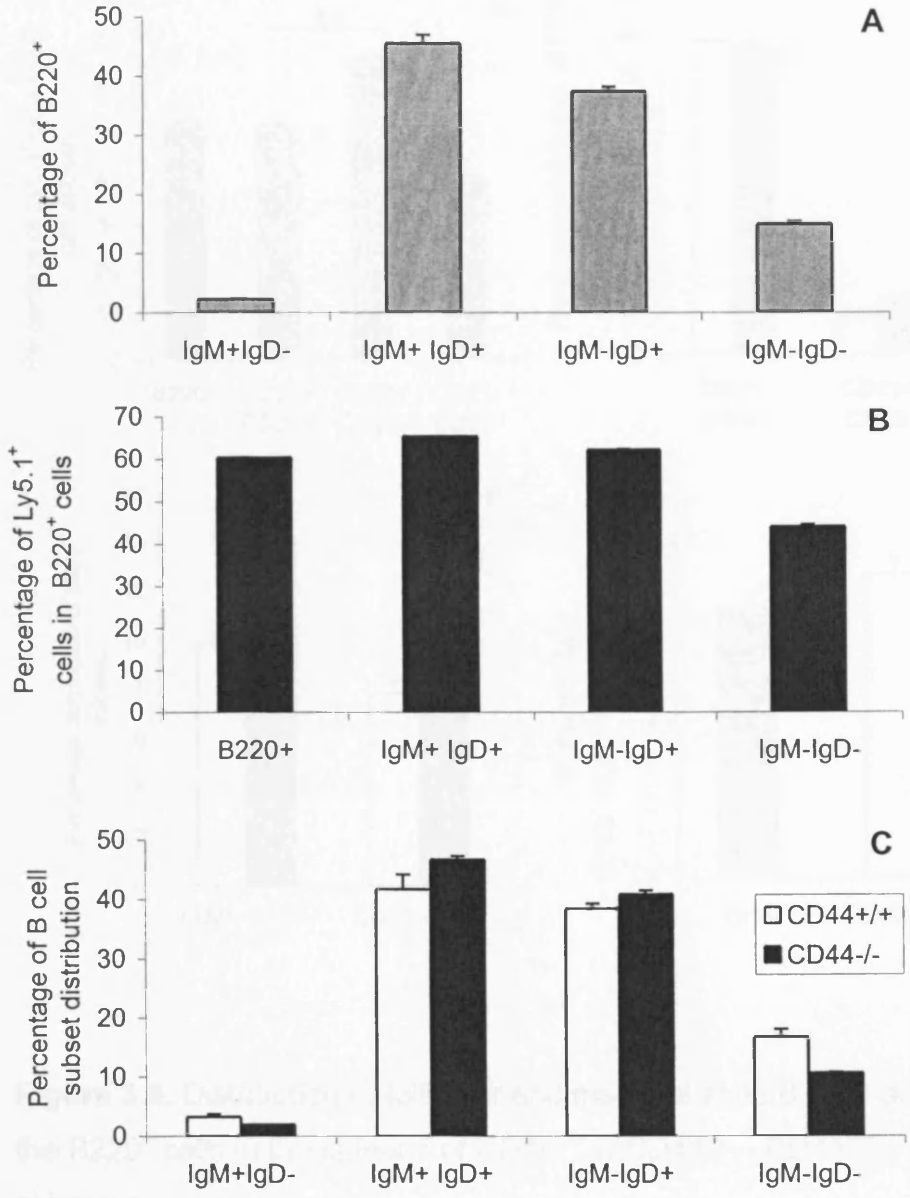

Figure 3.8. Distribution of sIgM$^+$ and sIgD$^+$ B cells in the lymph nodes of CD44$^{+/+}$ & CD44$^{-/-}$→ CD44$^{+/+}$ chimeras.

A. Distribution of B cells subsets defined by sIgM and sIgD expression in the B220$^+$ population of the lymph nodes. B. Contribution of CD44$^{-/-}$ cells to each subset of B220$^+$ cells as defined using sIgM and sIgD staining. C. Distribution of sIgM and sIgD staining among CD44$^{-/-}$ or CD44$^{+/+}$ B220$^+$ cells. Data are mean ± s.e.m. obtained from 1 group of 2 mice.

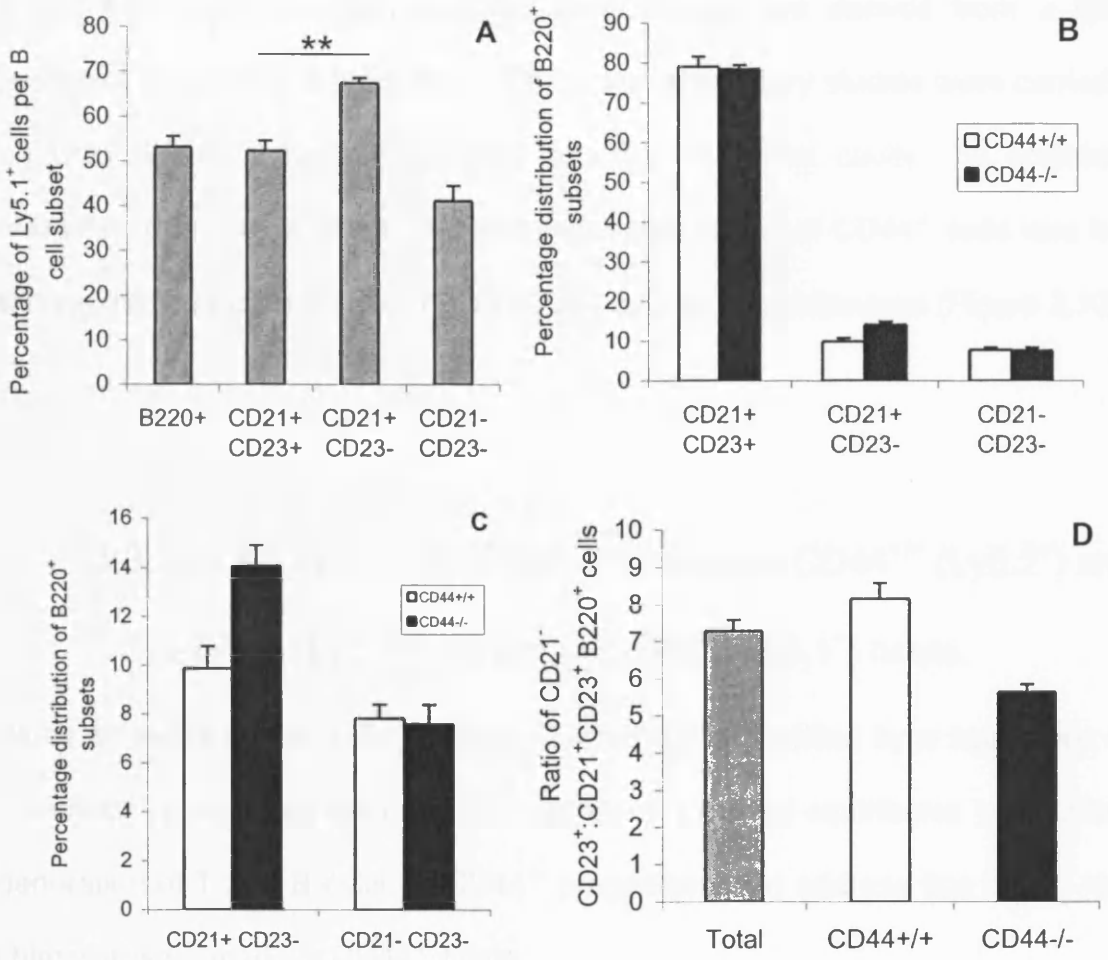

Figure 3.9. Distribution of follicular and marginal zone B cells among the $B220^+$ cells in the spleens of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A. CD21 and CD23 expression in the $B220^+$ population of the spleen. B. Contribution of $CD44^{-/-}$ cells to each subset of $B220^+$ cells as deficined by CD21 and CD23 staining. C. Alternative scale of (B) focusing on the CD23- populations. D. The ratio of $CD21^+$ $CD23^+$: $CD21^+$ $CD23^-$ cells among total, $CD44^{-/-}$ and $CD44^{+/+}$ $B220^+$ cells. Data are mean ± s.e.m. obtained from 1 group of 3 mice. P values, 2-sample t test; **, $P<0.01$

It has been proposed that marginal zone B cells are derived from a similar precursor to that of B-1 cells (227). Therefore, preliminary studies were carried out on $CD5^+$ $B220^+$ B-1a cells isolated from the peritoneal cavity. In contrast to marginal zone B cells, it was found that the percentage of $CD44^{-/-}$ cells was lower among the B-1a cells than non B-1a ($CD5^-$) cells in the peritoneum (Figure 3.10).

3.3.2. Phenotypic description of Chimeras: $CD44^{+/+}$ ($Ly5.2^+$) and $CD44^{-/-}$ ($Ly5.1^+$) donors → $CD44^{-/-}$ ($Ly5.1^+$) hosts.

Since adhesive interactions involving CD44 may be modified by a hyaluronic acid "sandwich" it was possible that host expression of CD44 contributes to the altered generation of T and B cells by $CD44^{-/-}$ progenitors. To address this issue, mixed chimeras were made in $CD44^{-/-}$ hosts.

Ly5.2 and $CD44^{-/-}$ mice were sacrificed and their bone marrow T cell depleted by antibody mediated complement cytotoxicity. The cells were mixed in a 1:1 ratio and injected into irradiated host $CD44^{-/-}$ mice (900 rads). Once immune reconstitution had occurred the mice were sacrificed, and their phenotype was analysed by FACS. As before, cells derived from the $CD44^{-/-}$ progenitor cells were identified using an anti-Ly5.1 (CD45.2) antibody.

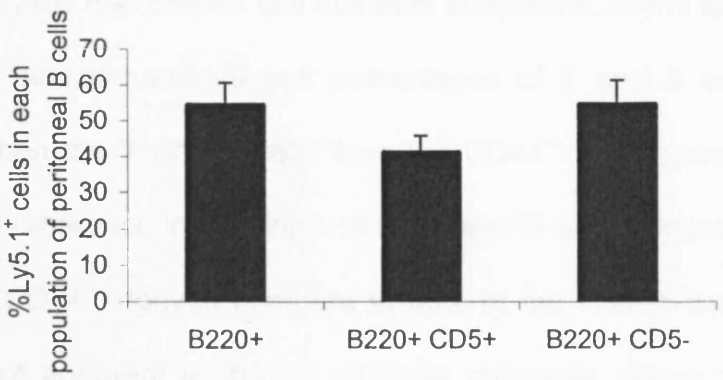

Figure 3.10. B220+ cells in the peritoneal cavity of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

Data are mean ± s.e.m. obtained from 1 group of 3 mice.

These mice also had normal cell numbers in spleens, thymi and LN (appendix 1), and overall similar numbers and percentages of T and B cells (Figures 3.11A, 3.12A). When the T and B cells from the $CD44^{-/-}$ host chimeras were analysed there was a difference in the origin of the T and B cells (Figure 3.11B). 24% of the T cells are $CD44^{-/-}$ derived compare to 35% of the B cells being $CD44^{-/-}$ derived. This was not apparent in the LN of these chimeras, where there was a similar contribution of $CD44^{-/-}$ cells to both the $CD3^{+}$ and $CD19^{+}$ populations. The bias in the spleen was also reflected in the B:T cell ratio, with the ratio of B:T cells among $CD44^{-/-}$ cells being 3.1, compared to 1.8 among total cells (Figure 3.11C).

The T cell populations in the spleen and LN were analysed, and it was observed that there was a difference in the ability of the $CD44^{-/-}$ progenitors to develop into $CD4^{+}$ versus $CD8^{+}$ T cells (Figure 3.13B, 3.14B). This is again highlighted when the $CD4^{+}$:$CD8^{+}$ T cell ratio is calculated. There were approximately 4 $CD4^{+}$ T cells per $CD8^{+}$ T cell in the $CD44^{-/-}$ population of the spleen compared to 3 $CD4^{+}$ T cells per $CD8^{+}$ T cell in the $CD44^{+/+}$ population in the spleen (Figure 3.13C). In the LN there were approximately 3 $CD4^{+}$ T cells per $CD8^{+}$ T cell in the $CD44^{-/-}$ population compared to 2 $CD4^{+}$ T cells per $CD8^{+}$ T cell in the $CD44^{+/+}$ population in the LN (Figure 3.14C). Of note, while the overall ratio of CD4:CD8 in the spleen of the $CD44^{+/+}$ hosts was 1.5, in the $CD44^{-/-}$ hosts it was 3, but in the LN of these different chimeras the CD4:CD8 T cell ratio was constant at 2.

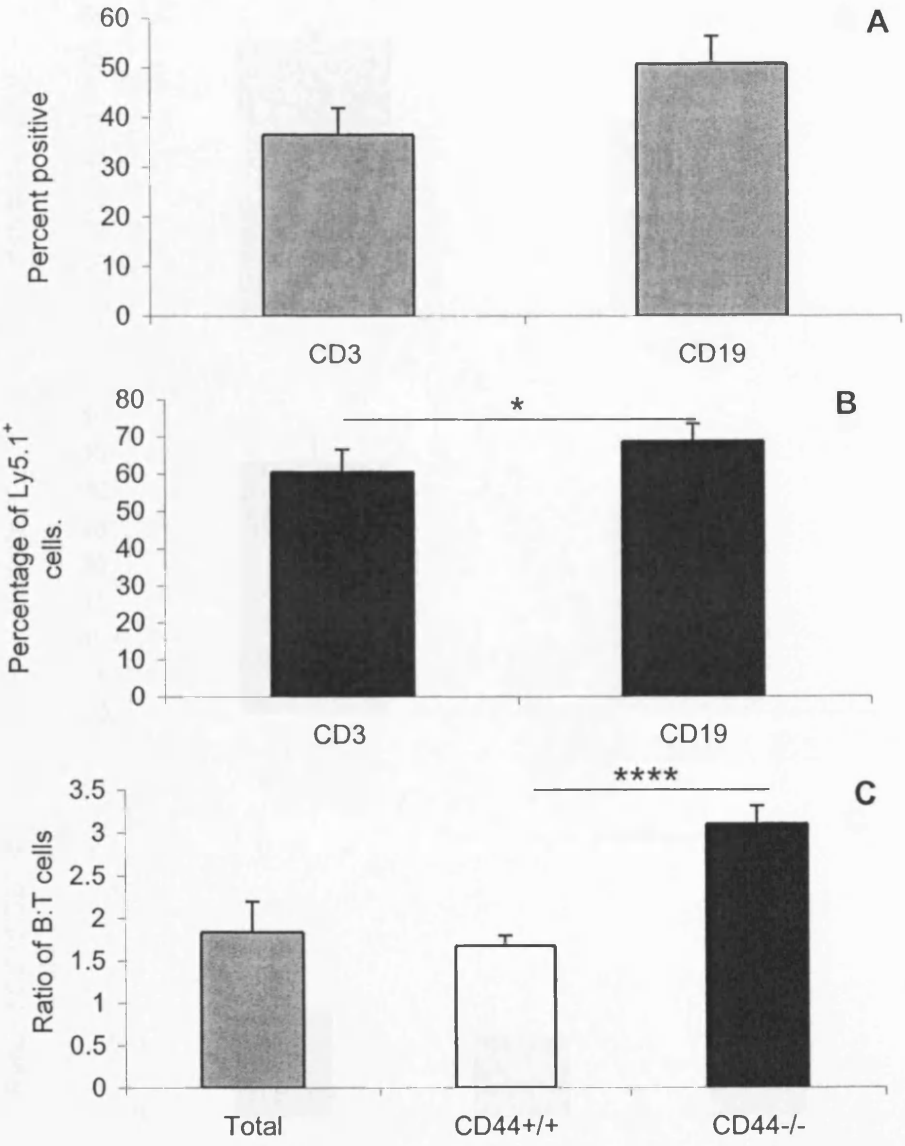

Figure 3.11. Analysis of T and B cells in the spleen of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{-/-}$ chimeras.

A. Percentages of T and B lymphocytes in spleen. B. Percentage of $Ly5.1^{+}$ cells in each splenic population. C. B:T cell ratio among total and $CD44^{-/-}$ populations. Data are mean ± s.e.m. obtained from 2 independent groups of chimeras, one group of 3 and one group of 8. P values, 2-sample t test; ****, $P<0.001$, *, $P<0.05$

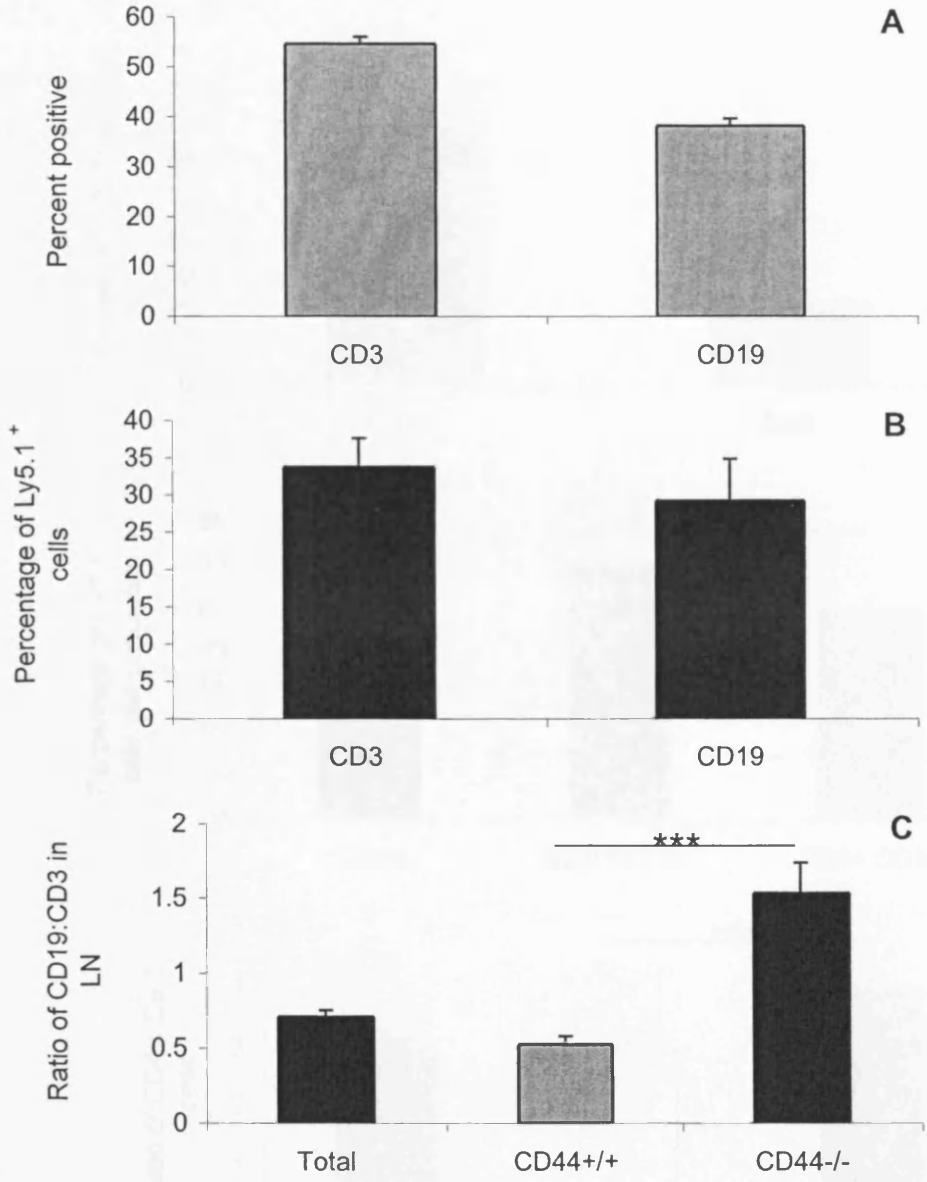

Figure 3.12. Analysis of T and B cells in lymph nodes of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{-/-}$ chimeras.

A. Percentages of T and B lymphocytes. B. Percentage of $Ly5.1^{+}$ cells in each population. C. B:T cell ratio among total, $CD44^{+/+}$ and $CD44^{-/-}$ populations. Data are mean ± s.e.m obtained from 2 independent groups of chimeras, one group of 2 and one group of 7. P values, 2-sample t test, ***, $P<0.005$

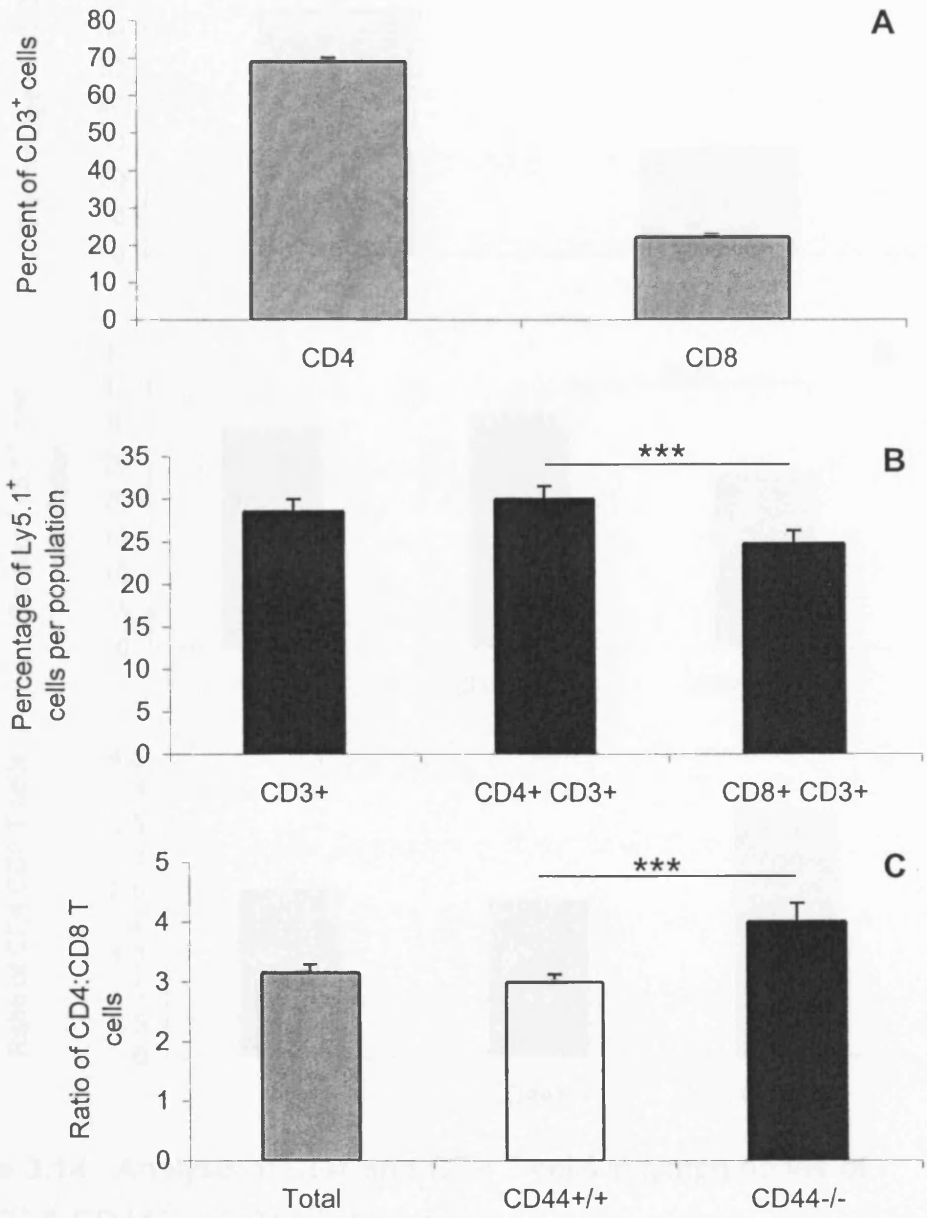

Figure 3.13. Analysis of CD4 and CD8 T cells in spleens of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{-/-}$ chimeras.

A. Percentages of $CD4^+$ and $CD8^+$ $CD3^+$ cells. B. Percentage of $Ly5.1^+$ cells in each splenic population. C. CD4:CD8 cell ratio among total, $CD44^{-/-}$ and $CD44^{+/+}$ populations. Data are mean ± s.e.m. obtained from 2 groups of independent chimeras, one group of 3 and one group of 8. P values, 2-sample T test; ***, $P<0.005$

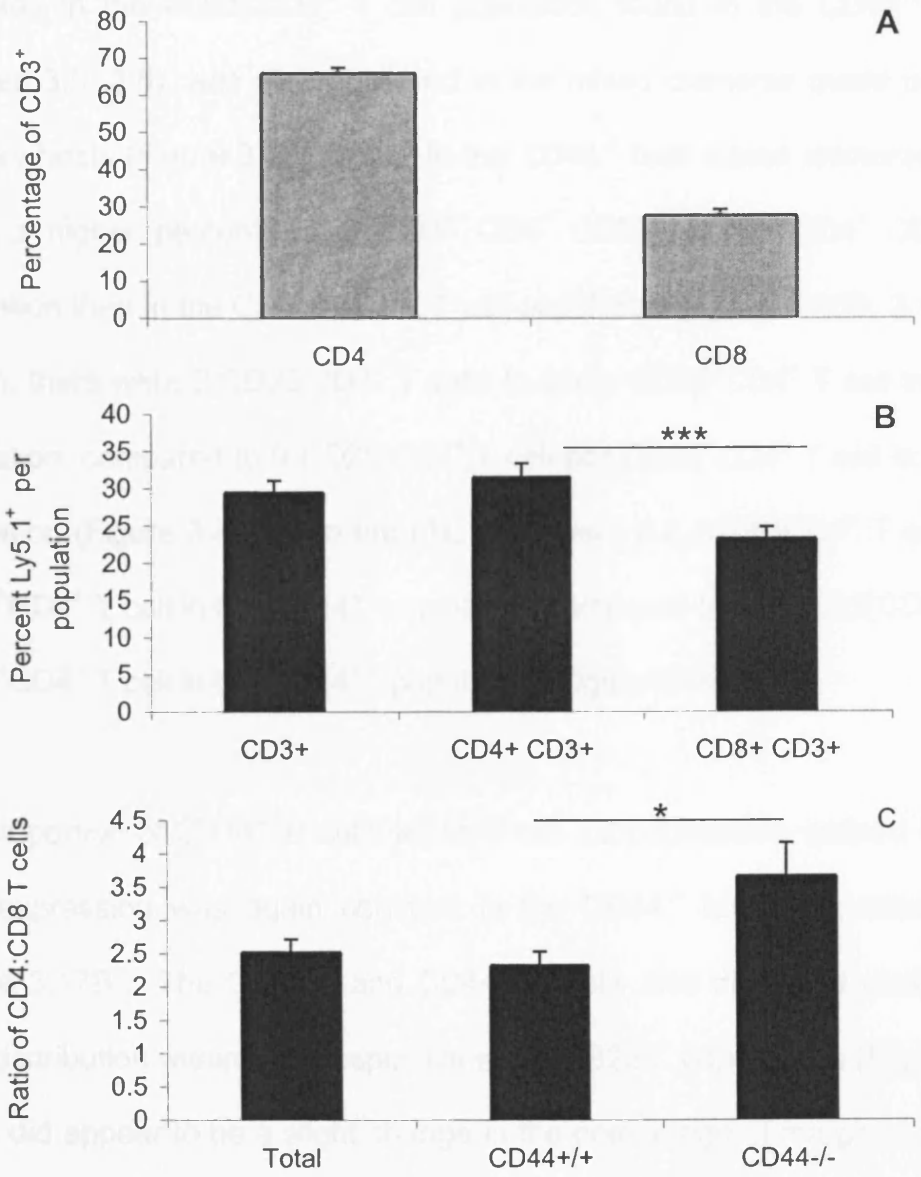

Figure 3.14. Analysis of CD4 and CD8 T cells in lymph nodes of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{-/-}$ chimeras.

A. Total percentages of $CD4^+$ and $CD8^+$ $CD3^+$ cells in lymph nodes. B. Percentage of $Ly5.1^+$ cells in each T cell subset. C. CD4:CD8 cell ratio among total, $CD44^{-/-}$ and $CD44^{+/+}$ populations. Data are mean ± s.e.m. obtained from 2 groups of independent chimeras, one group of 3 and one group of 8. P values, 2-sample t test; *, $P<0.05$, ***, $P<0.005$

The bias in the $CD25^+CD4^+$ T cell population found in the $CD44^{+/+}$ host mice (Figures 3.5, 3.6), was also observed in the mixed chimeras made using $CD44^{-/-}$ mice as hosts (Figure 3.15, 3.16). In the $CD44^{-/-}$ host mixed chimeras, there was again a higher percentage of $CD3^+$ $CD4^+$ $CD25^+$ in the $CD4^+$ $CD44^{-/-}$ T cell population than in the $CD4^+CD44^{+/+}$ T cell population (Figure 3.15B, 3.16B). In the spleen, there were 3 $CD25^-CD4^+$ T cells to every $CD25^+CD4^+$ T cell in the $CD44^{-/-}$ population, compared to 9 $CD25^-CD4^+$ T cell per $CD25^+CD4^+$ T cell in the $CD44^{+/+}$ population (Figure 3.15C). In the LN, there were 2.5 $CD25^-CD4^+$ T cells to every $CD25^+CD4^+$ T cell in the $CD44^{-/-}$ population, compared to 6.5 $CD25^-CD4^+$ T cell per $CD25^+CD4^+$ T cell in the $CD44^{+/+}$ population (Figure 3.16C).

The proportion of $CD44^{-/-}$ B cells within B cell subpopulations defined by sIgM and sIgD expression was again constant in the $CD44^{-/-}$ host mice mixed chimeras (Figure 3.17B). The $CD44^{+/+}$ and $CD44^{-/-}$ B cells also displayed similar sIgD and sIgM distribution within their respective splenic $B220^+$ populations (Figure 3.17C). There did appear to be a slight change in the percentage of marginal zone B cells, (Figure 3.18). There was an increase in the percentage of $CD44^{-/-}$ cells present among the marginal zone B cells compared to the amount found in the follicular B cell population although this is not statistically significant (Figure 3.18B). This again is explained by a statistically significantly higher marginal zone frequency in the $CD44^{-/-}$ B cell population, 14% in the $CD44^{-/-}$ B cell population compared to 12% in the $CD44^{+/+}$ B cell population (Figure 3.18C).

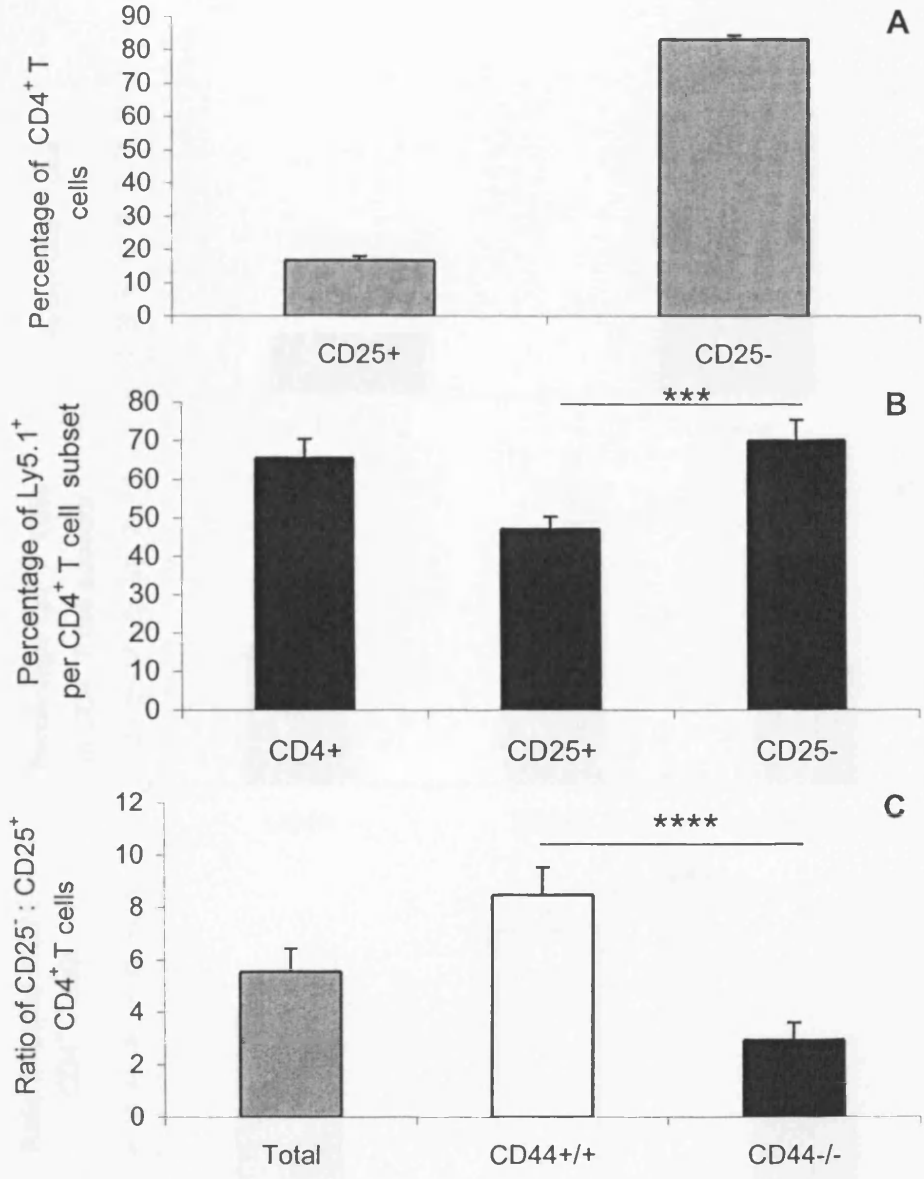

Figure 3.15. Analysis of CD4 subsets in spleens of $CD44^{+/+}$ & $CD44^{-/-}$ → $CD44^{-/-}$ chimeras.

A. Percentages of $CD25^{+}$ and $CD25^{-}$ $CD4^{+}$ splenic T cells. B. Percentage of $Ly5.1^{+}$ cells in each CD4 T cell subset. C. The $CD25^{-}$:$CD25^{+}$ $CD4^{+}$ T cell ratio among total, $CD44^{-/-}$ and $CD44^{+/+}$ populations. Data are mean ± s.e.m. obtained from 1 groups of 8 mice. P values, 2-sample t test; ***, P<0.005, ****, P<0.001.

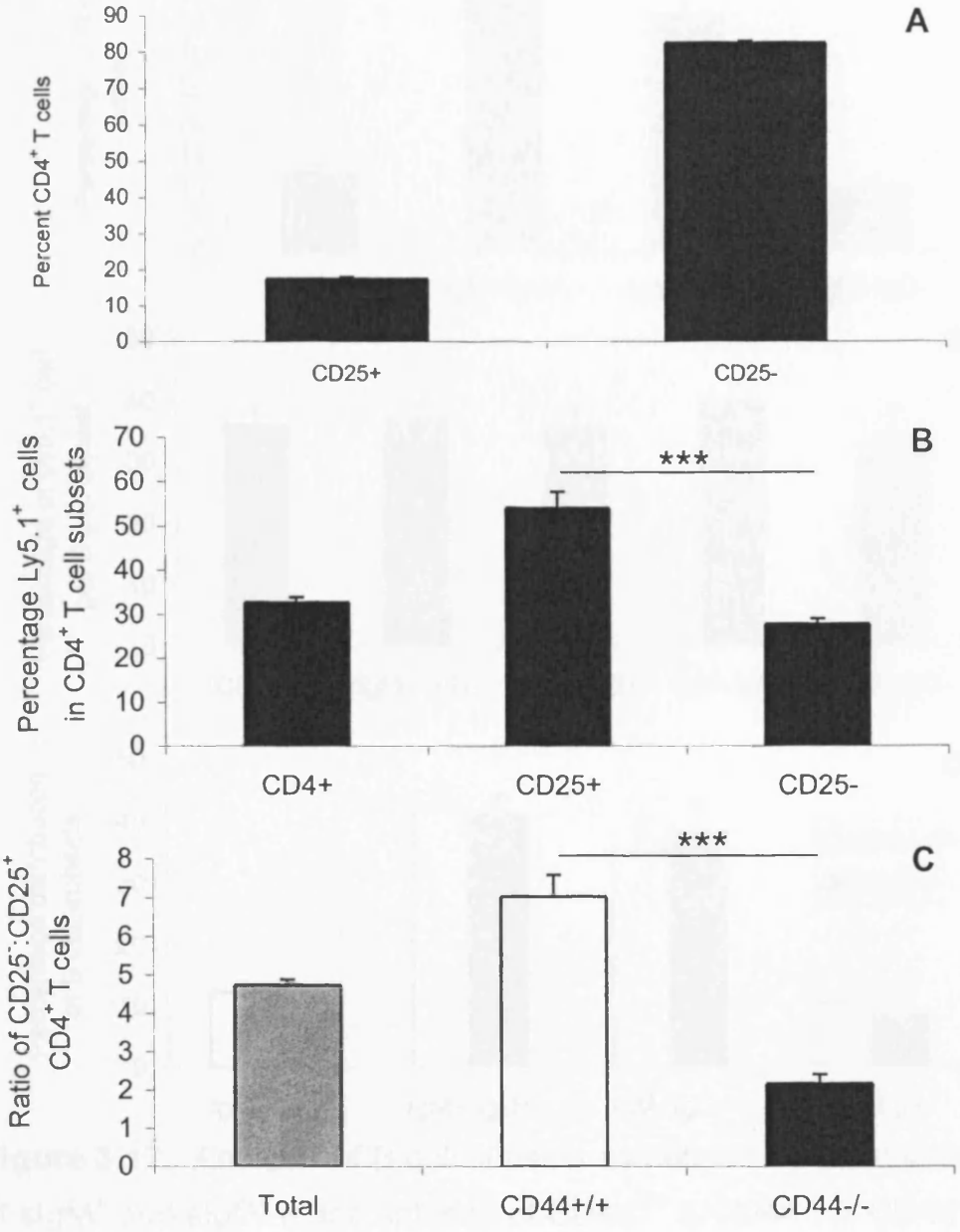

Figure 3.16. Analysis of CD4 subsets in lymph nodes of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{-/-}$ chimeras.

A. Percentages of $CD25^+$ and $CD25^-$ $CD4^+$ $CD3^+$ cells. B. Percentage of $Ly5.1^+$ cells in each CD4 T cell subset. C. The $CD25^-$:$CD25^+$ $CD4^+$ T cell ratio, among the total, $CD44^{-/-}$ and $CD44^{+/+}$ populations. Data are mean ± s.e.m. obtained from 1 group of 5 mice. P values, 2 sample t test; ***, $P<0.005$

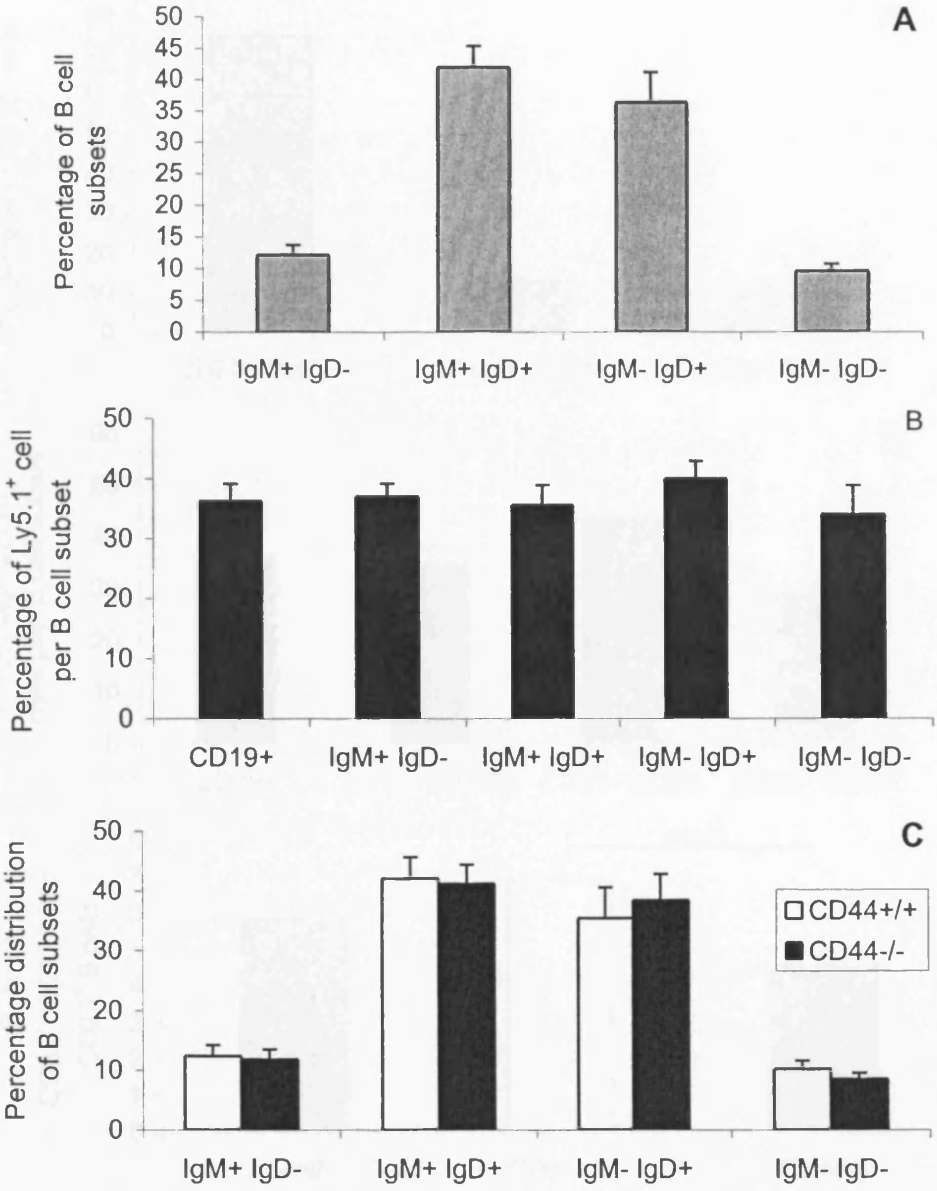

Figure 3.17. Analysis of B cell subsets as defined by the distribution of sIgM^{+} and sIgD^{+} in the spleens of CD44$^{+/+}$ & CD44$^{-/-}$→ CD44$^{-/-}$.

A. Distribution of B cells subsets defined by sIgM and sIgD expression in the CD19^{+} population of the spleen. B. Contribution of CD44$^{-/-}$ cells to each subset of CD19^{+} cells as defined using sIgM and sIgD staining. C. Distribution of sIgM and sIgD staining among CD44$^{-/-}$ or CD44$^{+/+}$ CD19^{+} cells. Data are mean ± s.e.m. obtained from 2 independent groups of chimeras: one group of 3 and one group of 8.

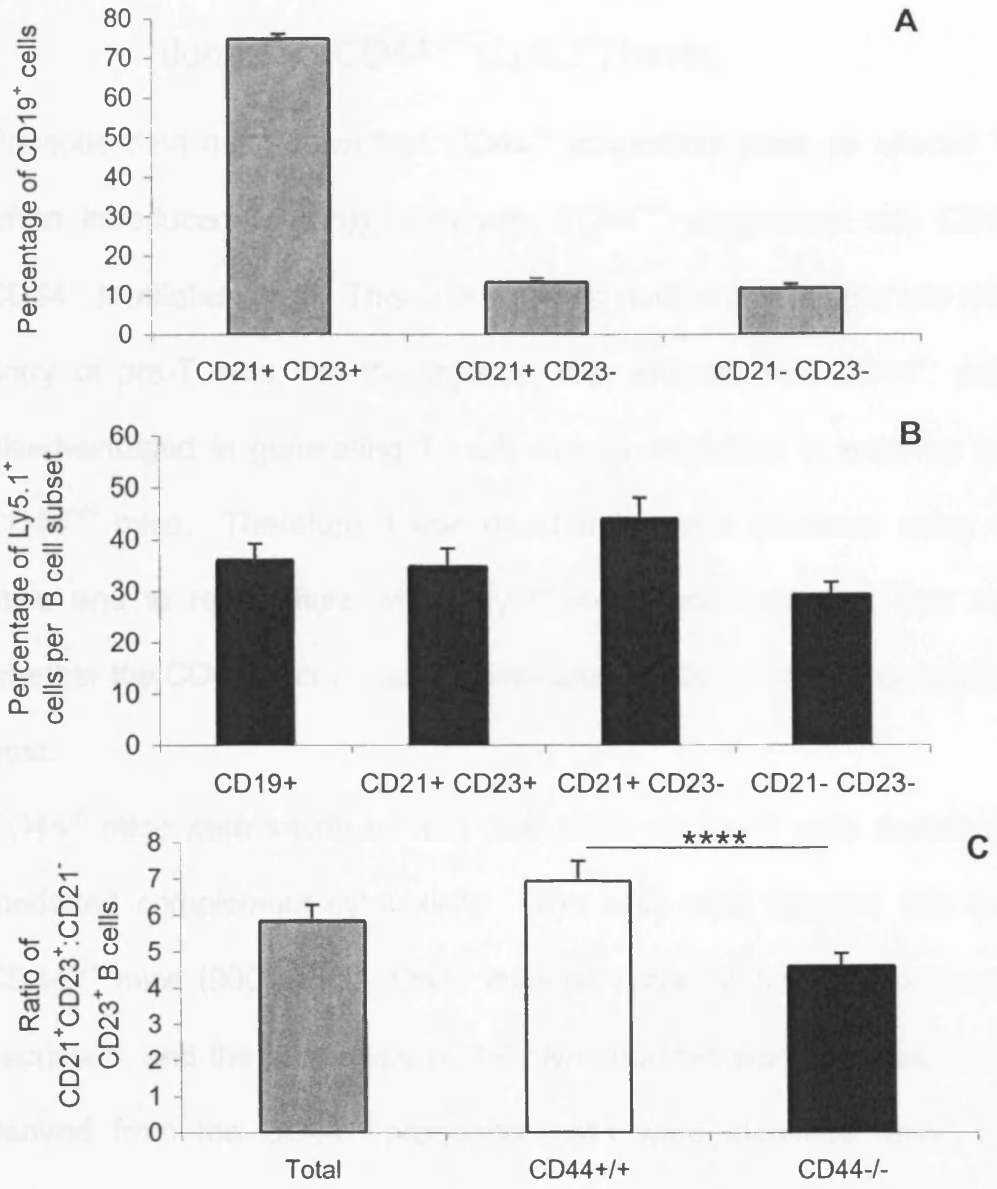

Figure 3.18. Distribution of follicular and marginal zone B cells among the $B220^+$ cells in the spleens of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{-/-}$ chimeras.

A. CD21 and CD23 expression in the $CD19^+$ population of the spleen. B. Contribution of $CD44^{-/-}$ cells to each subset of $CD19^+$ cells as defined by CD21 and CD23 staining. C. The ratio of $CD21^+$ $CD23^+$: $CD21^+$ $CD23^-$ cells among total, $CD44^{-/-}$ and $CD44^{+/+}$ $B220^+$ cells. Data are mean ± s.e.m. obtained from 2 independent groups of chimeras: one group of 3 and one group of 8. P values, 2-sample t test; ****, $P<0.001$

3.3.3. Phenotypic description of Chimeras: $CD44^{-/-}$ ($Ly5.1^{+}$) donors → $CD44^{+/+}$ ($Ly5.2^{+}$) hosts.

Previous data has shown that $CD44^{-/-}$ progenitors have an altered T:B cell ratio when introduced in conjunction with $CD44^{+/+}$ progenitors into $CD44^{+/+}$ but not $CD44^{-/-}$ irradiated hosts. This and previous studies indicating a role for CD44 in the entry of pre-T cells into the thymus, may indicate that $CD44^{-/-}$ progenitors are disadvantaged in generating T cells due to difficulties in entering the thymus of $CD44^{+/+}$ mice. Therefore it was decided to make chimeras using $CD44^{+/+}$ host mice and to reconstitute with only $CD44^{-/-}$ bone marrow. This will determine whether the $CD44^{-/-}$ bone marrow cells are capable of fully reconstituting a $CD44^{+/+}$ host.

$CD44^{-/-}$ mice were sacrificed and their bone marrow T cells depleted by antibody mediated complement cytotoxicity. The cells were injected into irradiated host $CD44^{+/+}$ mice (900 rads). Once immune reconstitution had occurred they were sacrificed, and the phenotype of their lymphocytes was analysed by FACS. Cells derived from the $CD44^{-/-}$ progenitor cells were identified using the anti-Ly5.1 (CD45.2) antibody.

These mice again had normal spleens, thymi and LN, and numbers (appendix 1) and percentages of total T and B cells that were comparable to that found in all of the other chimeras (compare Figures 3.1A & 3.2A, 3.11A & 3.12A, 3.19A & 3.20A). Notably, although essentially 100% of the B cells were derived from $CD44^{-/-}$ progenitors, there was a significant number of host T cells remaining. Only about 70% of T cells in the spleen were $CD44^{-/-}$ (Figure 3.19B), compared to 85% of the T cells in the LN (Figure 3.20B). This was due to the relative radioresistance of

mature T cells which are predominately found in the spleen (228). Nevertheless, it was evident that $CD44^{-/-}$ progenitors were capable of generating T cells in irradiated $CD44^{+/+}$ hosts.

Similar proportions of $CD4^+$ and $CD8^+$ T cells were $CD44^{-/-}$ in the $CD44^{+/+}$ hosts (Figures 3.21, 3.22), indicating that there was no bias in generating CD4 versus CD8 T cells in this setting, though there was a significantly larger amount of radioresistant $CD8^+$ rather than $CD4^+$ T cells. Interestingly, although there were a significant proportion of radioresistant T cells in these chimeras, in the spleen there was a higher proportion of radioresistant $CD25^+CD4^+$ than $CD25^-CD4^+$ host T cells (Figure 3.23B). While this difference was small, it enhances the ability of the $CD44^{-/-}$ progenitors to produce $CD25^+CD4^+$ T cells which was observed in other chimeras.

All splenic B cell subsets were present in these chimeras and very few radioresistant host B cells were detected (Figures 3.25, 3.26). While subset composition varied between different chimeras, there was a trend towards an increased proportion of both IgM^-IgD^+ cells and marginal zone ($CD21^+CD23^-$) cells compared to the control chimeras described next (Figure 3.27). This is again consistent with the observations in mixed chimeras.

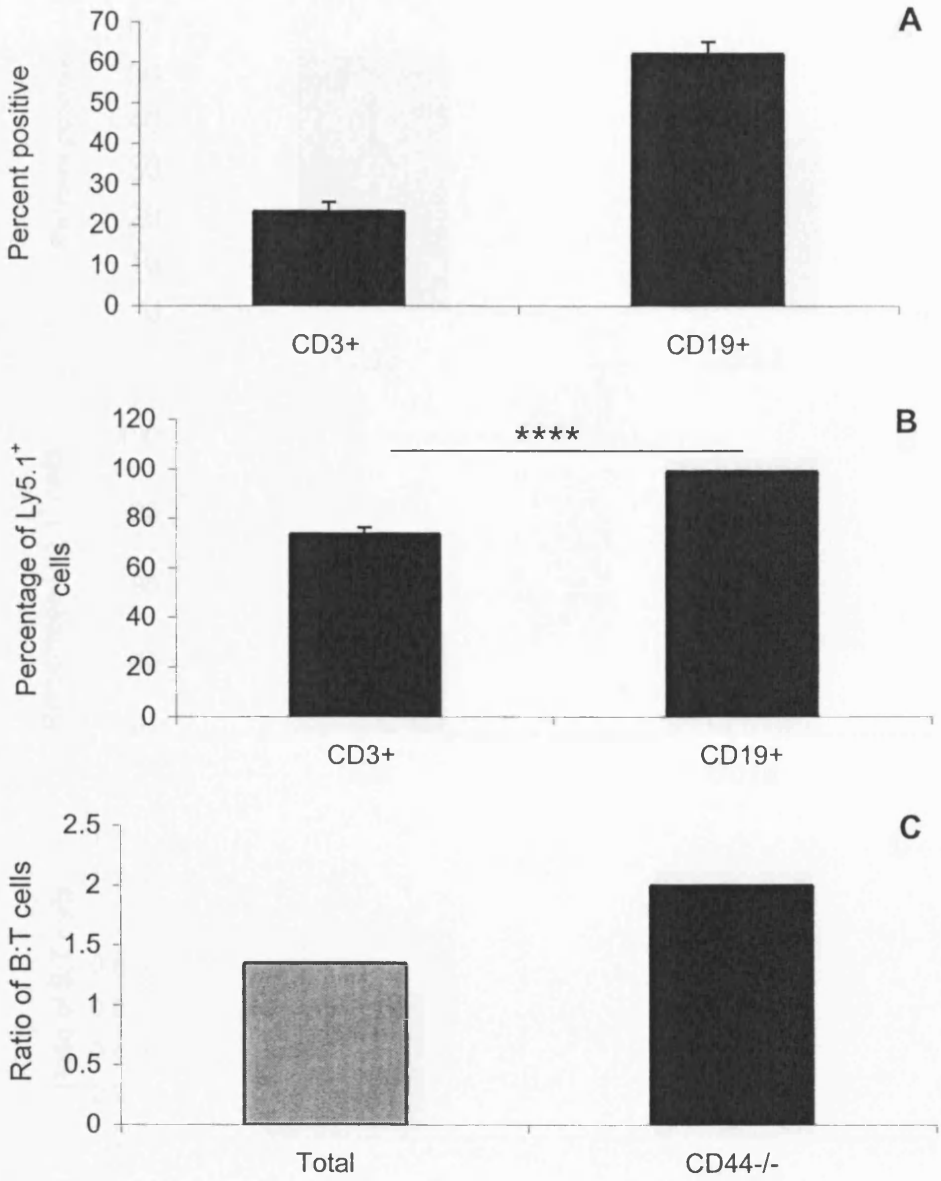

Figure 3.19. Analysis of B vs T cells in the spleen of $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A. Percentages of T and B cells in spleen. B. The percentage of donor $Ly5.1^{+}$ cells present in T and B cell populations. C. The B: T cell ratio among total and donor splenocytes. Data are mean ± s.e.m. obtained from 2 groups of mice: one group of 3 and one group of 4. P values, 2 sample t test; ****, $P<0.001$

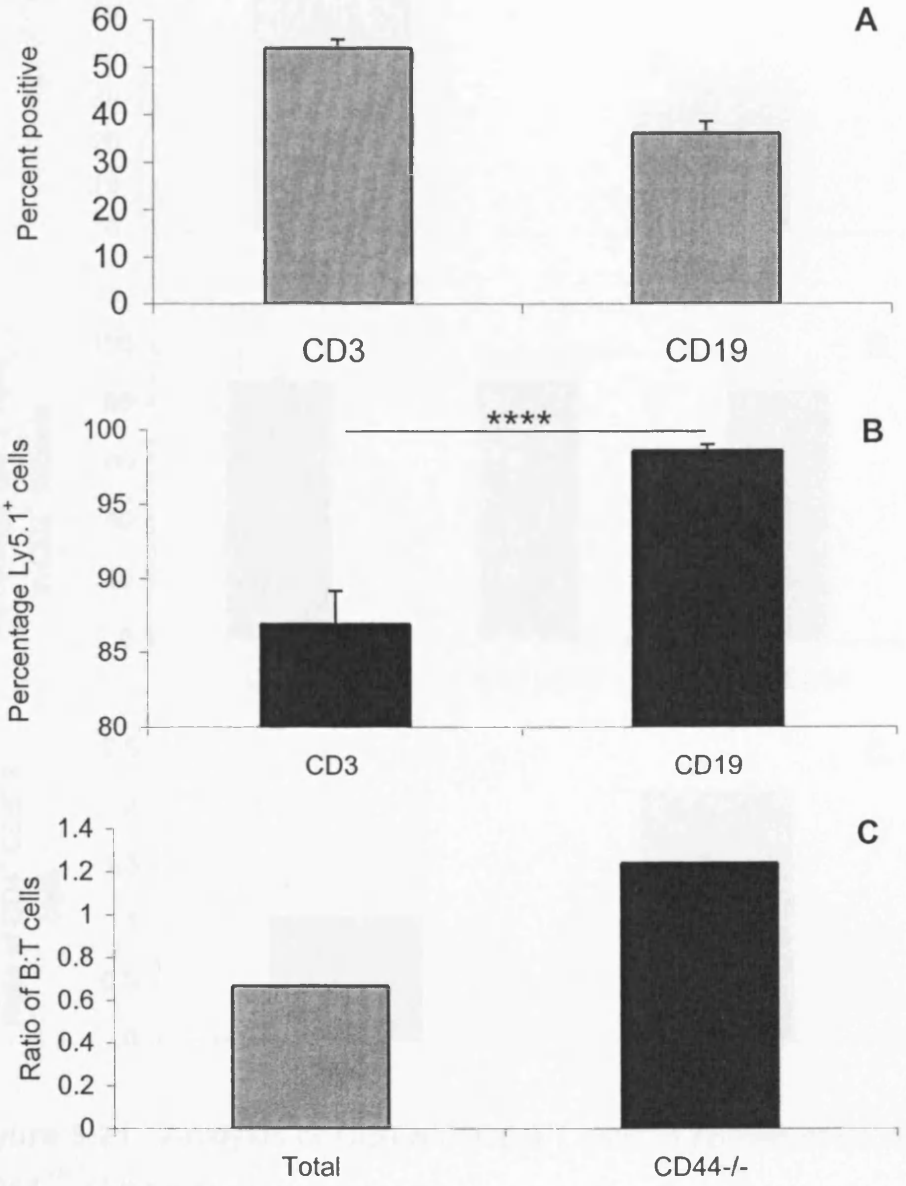

Figure 3.20. Analysis of B vs T cells in the lymph nodes of $CD44^{-/-}\rightarrow CD44^{+/+}$ chimeras.

A. Percentages of T and B cells in lymph node. B. The percentage of donor $Ly5.1^{+}$ cells present in T and B cell populations. C. The B: T cell ratio among total and donor lymphocytes. Data are mean ± s.e.m. obtained from 2 groups of mice: one group of 3 and one group of 4. P values, 2-sample t test; ****, $P<0.001$

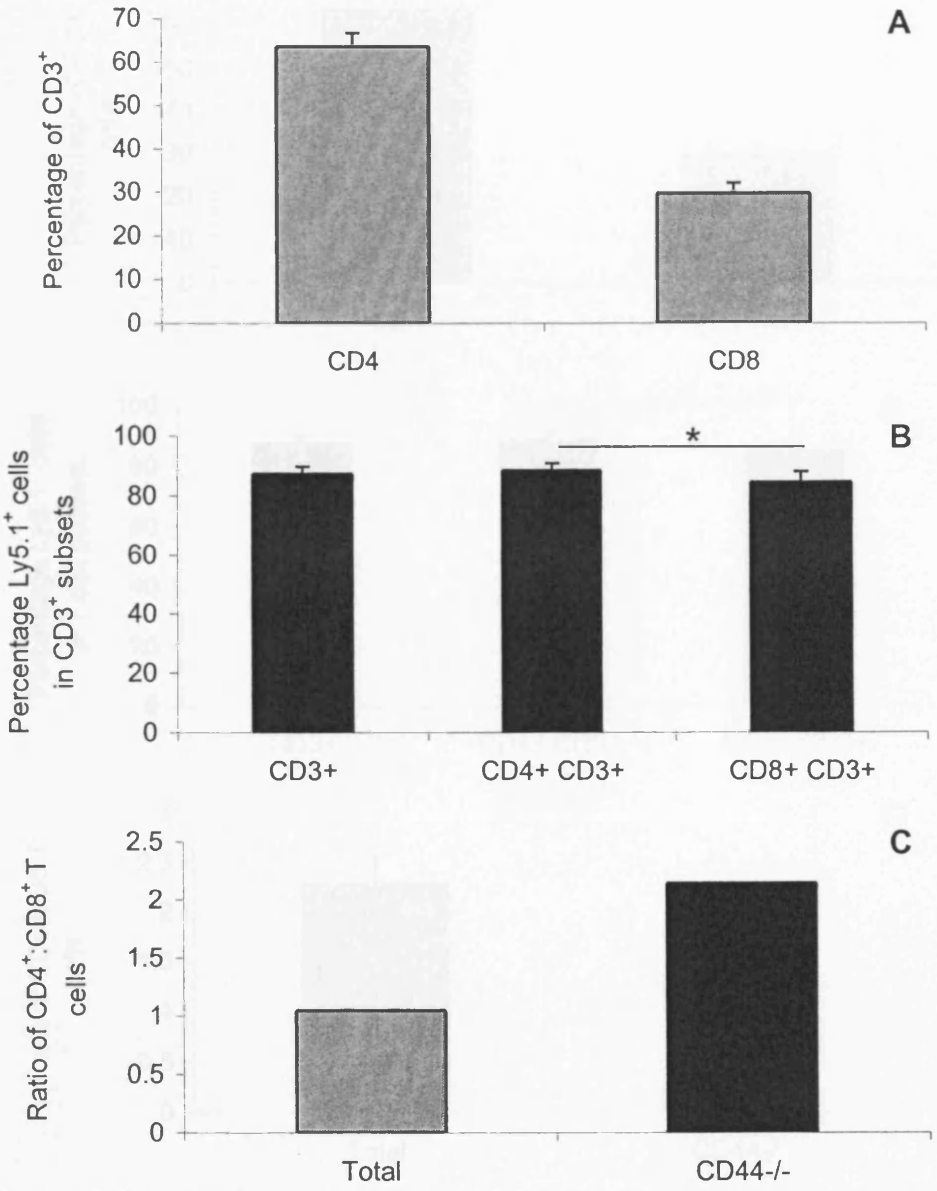

Figure 3.21. Analysis of CD4 and CD8 T cells in spleen of $CD44^{-/-}\rightarrow CD44^{+/+}$ chimeras.

A. Percentages of $CD4^+$ and $CD8^+$ $CD3^+$ cells. B. The percentage of $Ly5.1^+$ cells in each splenic population. C. The CD4:CD8 cell ratio among total and donor $CD44^{-/-}$ populations. Data are mean ± s.e.m. obtained from 2 groups of mice: one group of 3 and one group of 4. P values, 2-sample t test; *, $P<0.05$

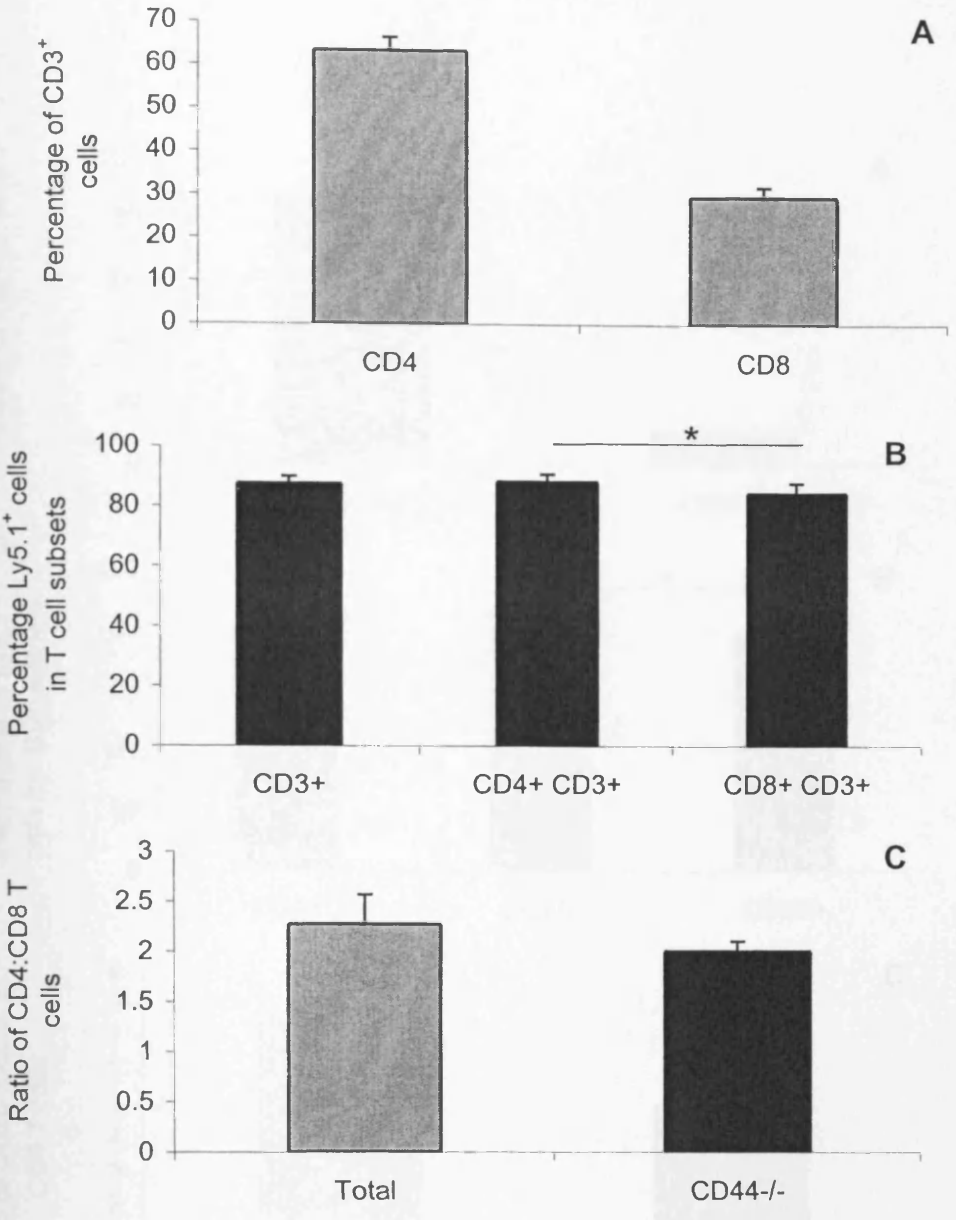

Figure 3.22. Analysis of CD4 and CD8 T cells in lymph nodes of $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A. Percentages of $CD4^+$ and $CD8^+$ $CD3^+$ cells. B. The percentage of $Ly5.1^+$ cells in each T cell subset population. C. The CD4:CD8 cell ratio among the total and donor population. Data are mean ± s.e.m. obtained from 2 groups of mice: one group of 3 and one group of 4. P values, 2 sample t test; *, $P<0.05$

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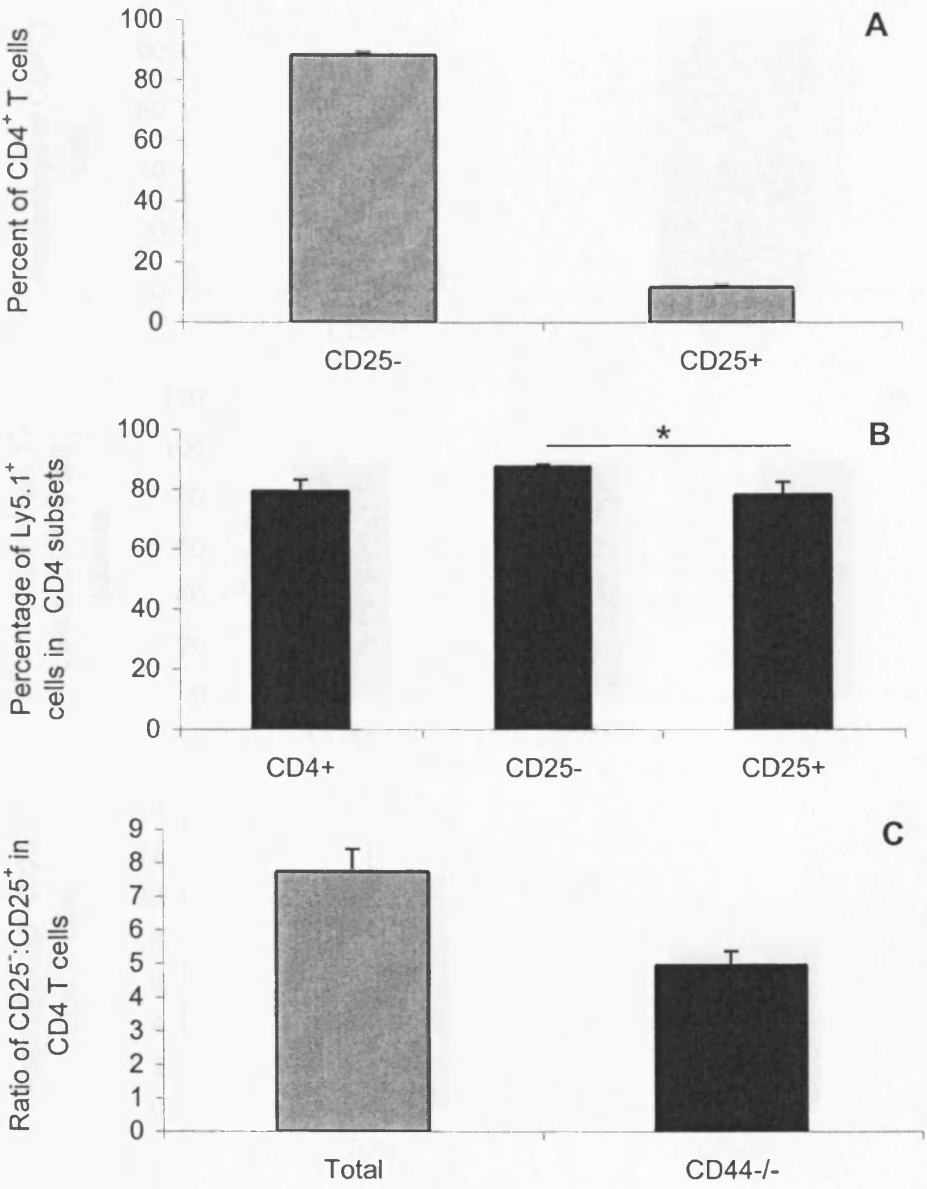

Figure 3.23. Analysis of CD4 subset in spleen of $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A. Percentages of $CD25^+$ and $CD25^-$ $CD4^+$ $CD3^+$ cells in spleen. B. Percentage of $Ly5.1^+$ cells in each CD4 subset. C. The $CD25^-$:$CD25^+$ $CD4^+$ T cell ratio among total and donor cells. Data are mean ± s.e.m. obtained from 1 group of 3 mice. P values, 2-sample t test; *, $P<0.05$

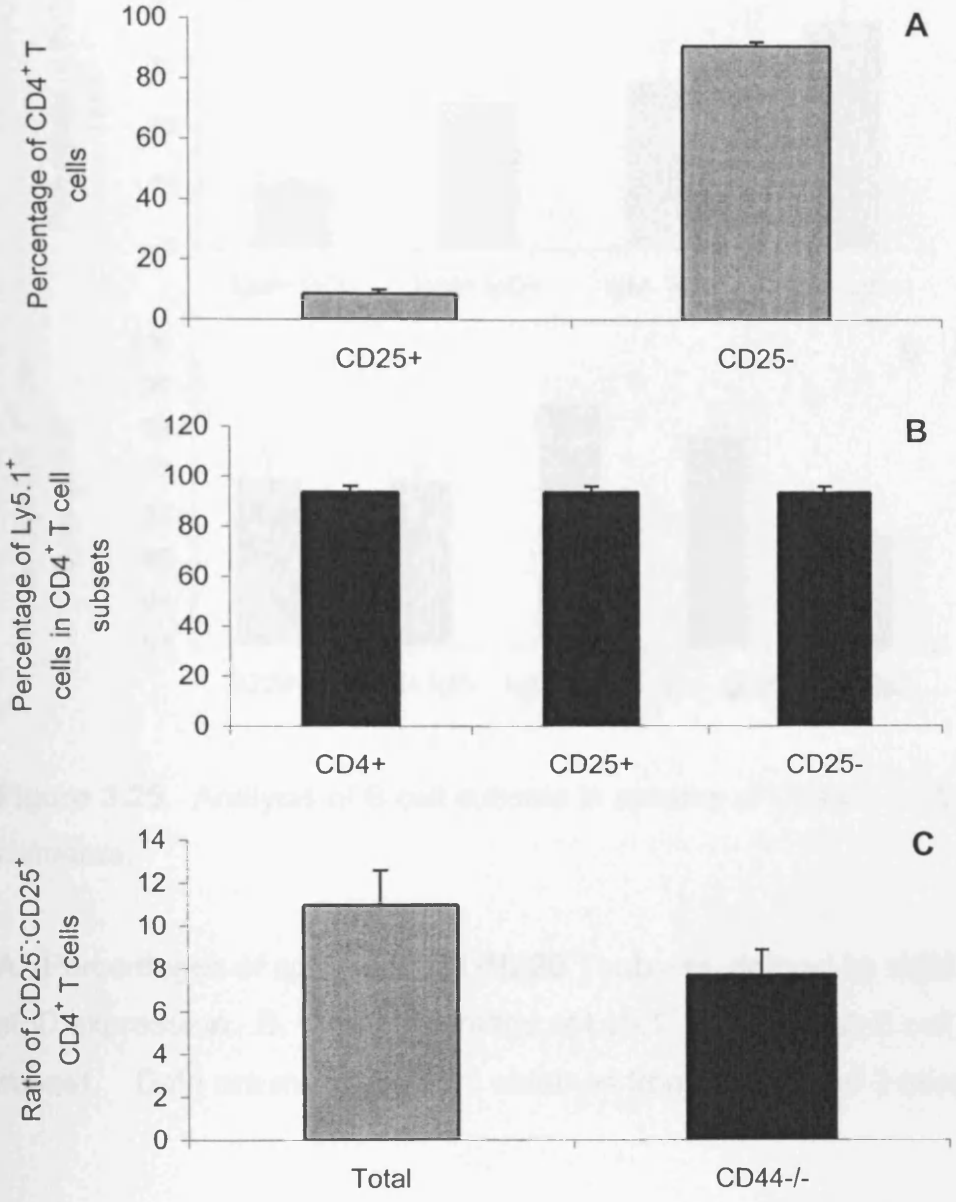

Figure 3.24. Analysis of CD4 subset in lymph nodes of $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A. Percentages of $CD25^+$ and $CD25^-$ $CD4^+$ $CD3^+$ cells in lymph nodes. B. Percentage of $Ly5.1^+$ cells in each CD4 subset. C. The $CD25^-$:$CD25^+$ $CD4^+$ T cell ratio among total and donor cells. Data are mean ± s.e.m. obtained from 1 group of 3 mice.

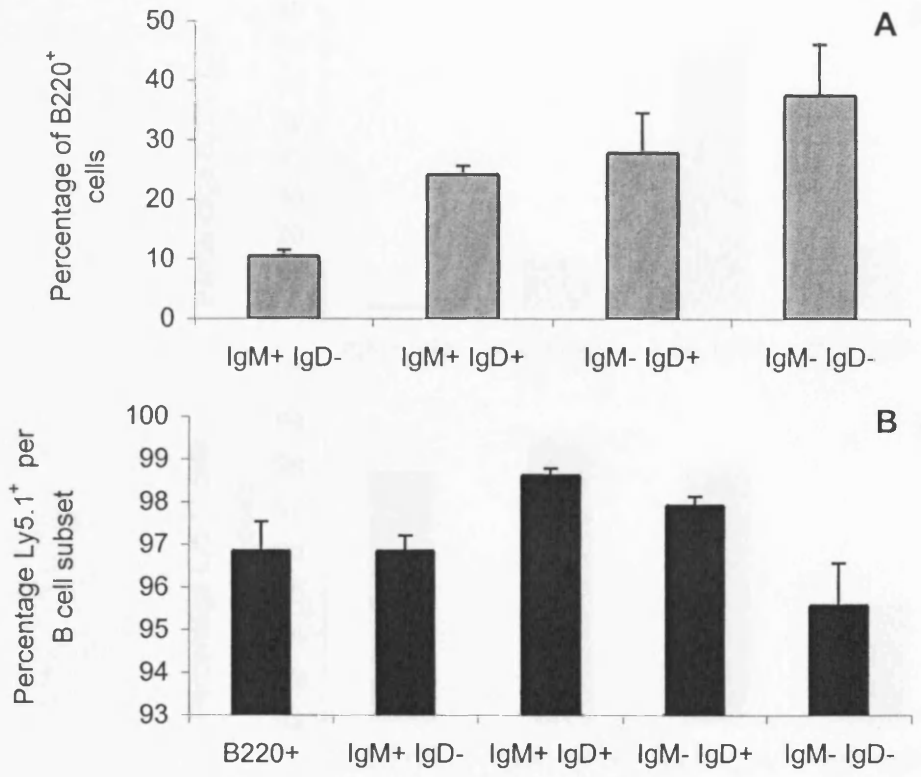

Figure 3.25. Analysis of B cell subsets in spleens of $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A. Percentages of splenic B cell ($B220^+$) subsets, defined by sIgM and sIgD expression. B. The percentage of $Ly5.1^+$ cells in each B cell subset. Data are mean ± s.e.m. obtained from 2 groups of 3 mice.

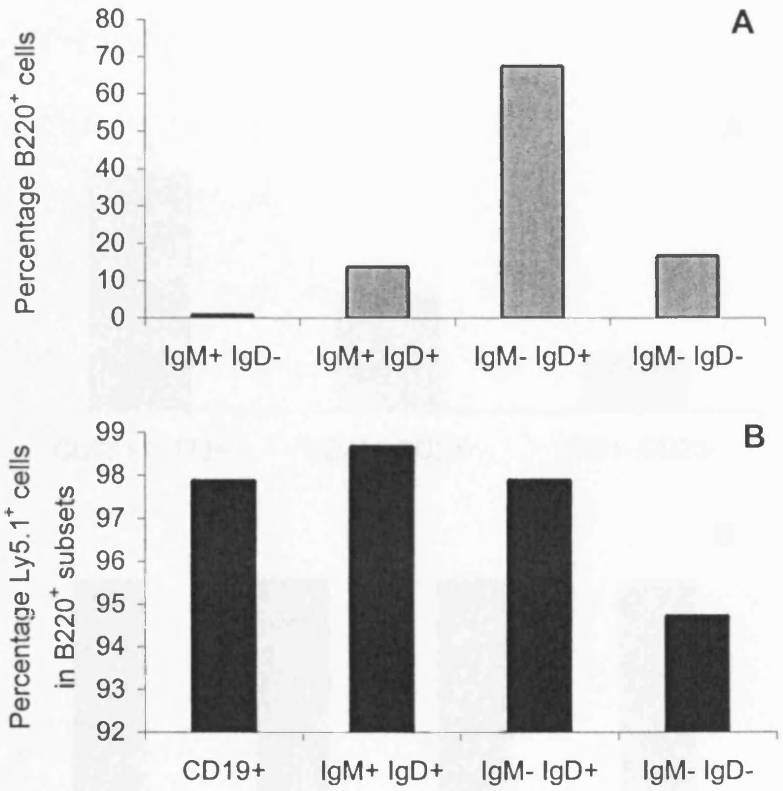

Figure 3.26. Analysis of B cell subsets in lymph nodes of $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A. Percentages of lymph node B cell ($B220^+$) subsets, defined by sIgM and sIgD expression. B. The percentage of $Ly5.1^+$ cells in each B cell subset. Data are mean ± s.e.m. obtained from 1 group of 4 mice.

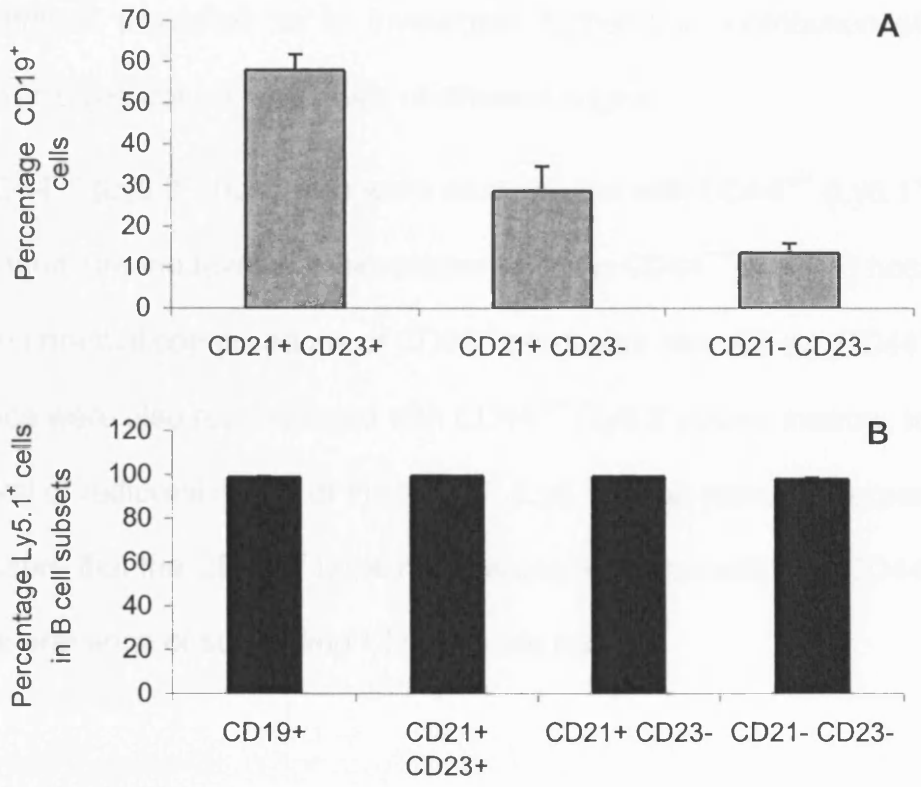

Figure 3.27. Distribution of follicular and marginal zone B cells among the $CD19^+$ cells in the spleens of $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A. Percentages of splenic B cells as defined by CD21 and CD23 expression. B. Contribution of $CD44^{-/-}$ cells to each subset of $CD19^+$ cells as defined by CD21 and CD23 staining. Data are mean ± s.e.m. obtained from 1 group of 4 mice.

3.3.4. Measurement of Radioresistance in chimeras

The presence of radioresistant host T cells in $CD44^{-/-}$ ($Ly5.1^{+}$) → $CD44^{+/+}$ ($Ly5.2^{+}$) chimeras prompted us to investigate further the contribution of radioresistance when using donors and hosts of different origins.

$CD44^{+/+}$ ($Ly5.2^{+}$) host mice were reconstituted with $CD44^{+/+}$ ($Ly5.1^{+}$) bone marrow, to measure the level of radioresistance of the $CD44^{+/+}$ ($Ly5.2^{+}$) host mice, without the potential complications of $CD44^{-/-}$ progenitor disabilities. $CD44^{-/-}$ ($Ly5.1^{+}$) host mice were also reconstituted with $CD44^{+/+}$ ($Ly5.2^{+}$) bone marrow, to measure the level of radioresistance of the $CD44^{-/-}$ ($Ly5.1^{+}$) host cells; this experiment will also ensure that the $CD44^{+/+}$ bone marrow can fully reconstitute a $CD44^{-/-}$ host, without the presence of supporting $CD44^{-/-}$ bone marrow.

3.3.4.1. Phenotypic description of Chimeras: $CD44^{+/+}$ ($Ly5.1^{+}$) → $CD44^{+/+}$ ($Ly5.2^{+}$).

$CD44^{+/+}$ (Ly5.1) mice were sacrificed and their bone marrow T cell depleted by antibody mediated complement cytotoxicity. T-depleted cells were injected into irradiated host $CD44^{+/+}$ (Ly5.2) mice (900 rads). Once immune reconstitution had occurred, mice were sacrificed, and the phenotype of their lymphocytes was analysed by FACS. Donor cells were identified using the anti-Ly5.1 (CD45.2) antibody.

Similar numbers (appendix 1) and percentages of total T and B cells, to those seen in the other chimeras were observed (Figures 3.28A & 3.29A, 3.1 & 3.2A, 3.11 & 3.12A and 3.19 & 3.20A). When the T and B cells from the spleen and LN of these chimeras were analysed there was again a significant amount of radioresistance in the T cell population but few radioresistant B cells (Figure 3.28B, 3.29B). Although the host mice were lethally irradiated with 900 rads, approximately 20% of the T cells in the spleen and 15% in the LN were radioresistant host cells. This compared to approximately 30% and 15% of T cells in the spleen and LN respectively when these mice were reconstituted with $CD44^{-/-}$ bone marrow (Figure 3.19B).

The T cell populations in the spleen and LN showed no difference in $CD44^{+/+}$ progenitors developing into either $CD4^+$ or $CD8^+$ T cells in the $CD44^{+/+}$ hosts.
In the spleen there no difference in the frequency of radioresistant cells among $CD4^+$ or $CD8^+$ host T cells, although in the LN there was a significant number of $CD8^+$ radioresistant T cells (Figure 3.30, 3.31). In the $CD4^+$ T cell populations, the $CD25^+CD4^+$ T cells were more resistant to irradiation than the $CD25^-$ $CD4^+$ T cells (Figure 3.32, 3.33).

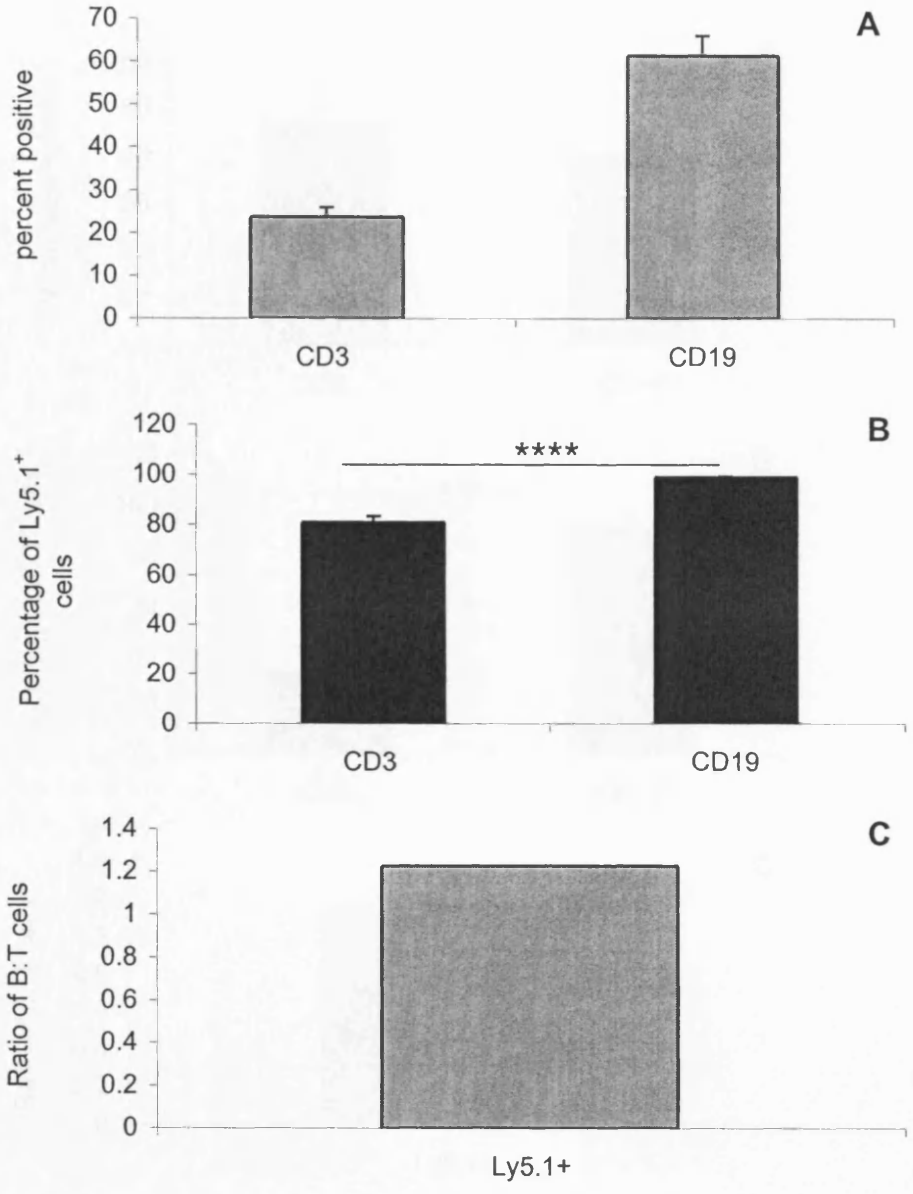

Figure 3.28. Analysis of B vs T cells in the spleen of $CD44^{+/+}$ (Ly5.1)→ $CD44^{+/+}$ (Ly5.2) chimeras.

A. Percentages of T and B cells in spleen. B. The percentage of donor $Ly5.1^+$ cells present in each T and B cell population. C. The B:T cell ratio among donor ($Ly5.1^+$) lymphocytes. Data are mean ± s.e.m. obtained from 2 groups of 4 mice. P values, 2-sample t test; ****, $P<0.001$

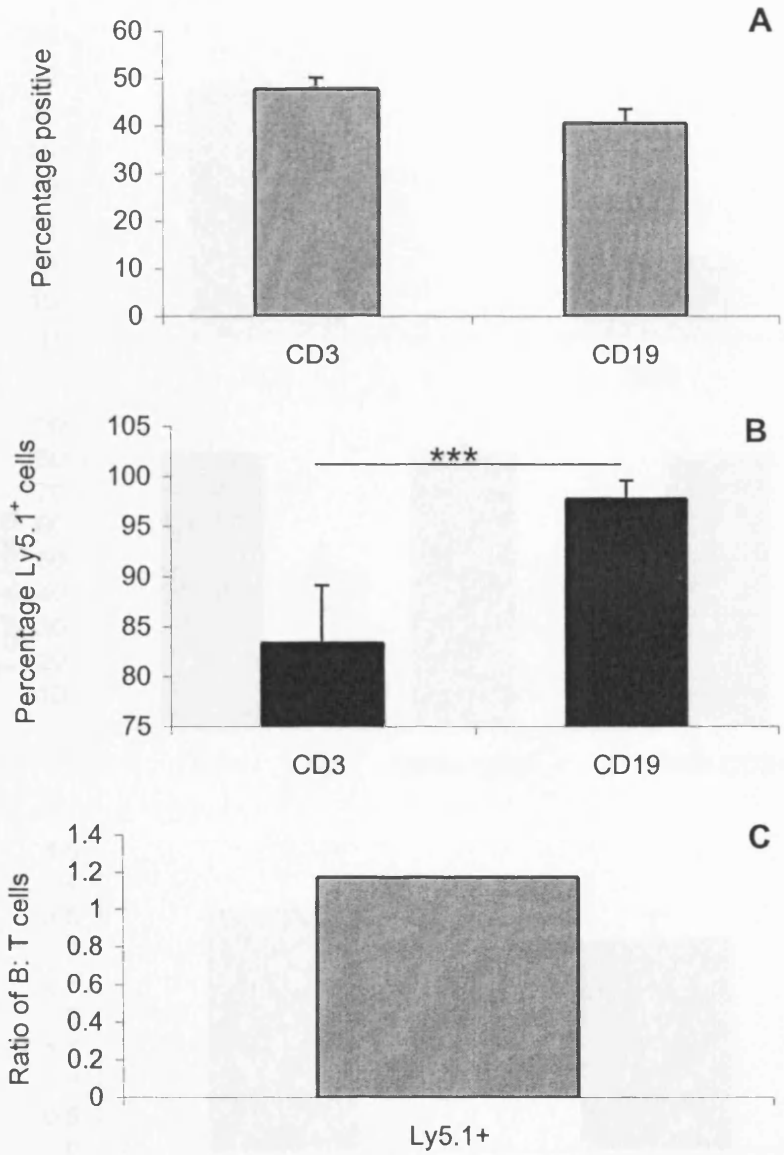

Figure 3.29. Analysis of B vs T cells in the lymph nodes of $CD44^{+/+}$ (Ly5.1)→ $CD44^{+/+}$ (Ly5.2) chimeras.

A. Percentages of T and B cells in spleen. B. The percentage of donor $Ly5.1^{+}$ cells present in T and B cell population. C. The B:T cell ratio among donor ($Ly5.1^{+}$) lymphocytes. Data are mean ± s.e.m. obtained from 2 groups of 4 mice. P values, 2-sample t test; ***, $P<0.005$

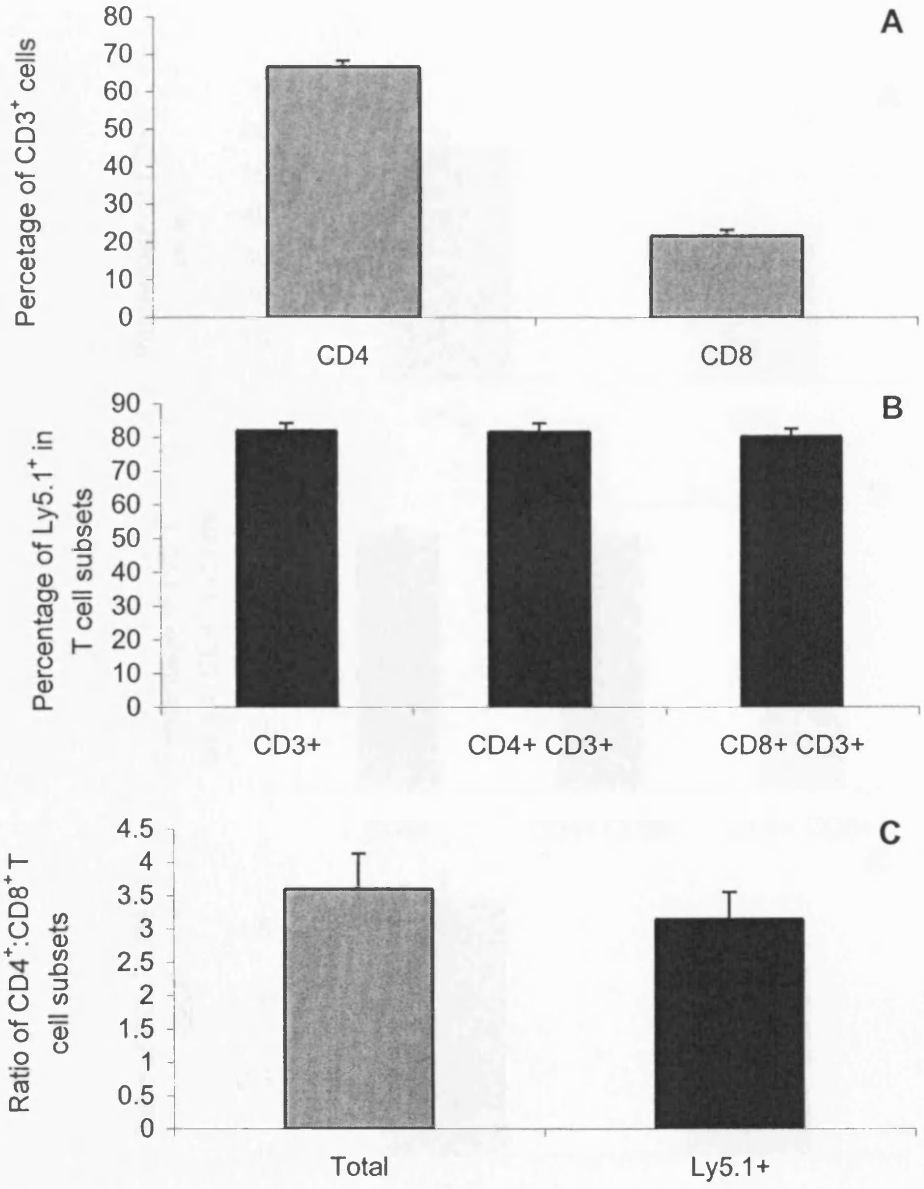

Figure 3.30. Analysis of CD4 and CD8 T cells in spleen of $CD44^{+/+}$ (Ly5.1) → $CD44^{+/+}$ (Ly5.2) chimeras.

A. Percentages of $CD4^+$ and $CD8^+$ $CD3^+$ cells. B. The percentage of $Ly5.1^+$ cells in each splenic population. The CD4:CD8 T cell ratio among total and donor cells. Data are mean ± s.e.m. obtained from 2 groups of 4 mice.

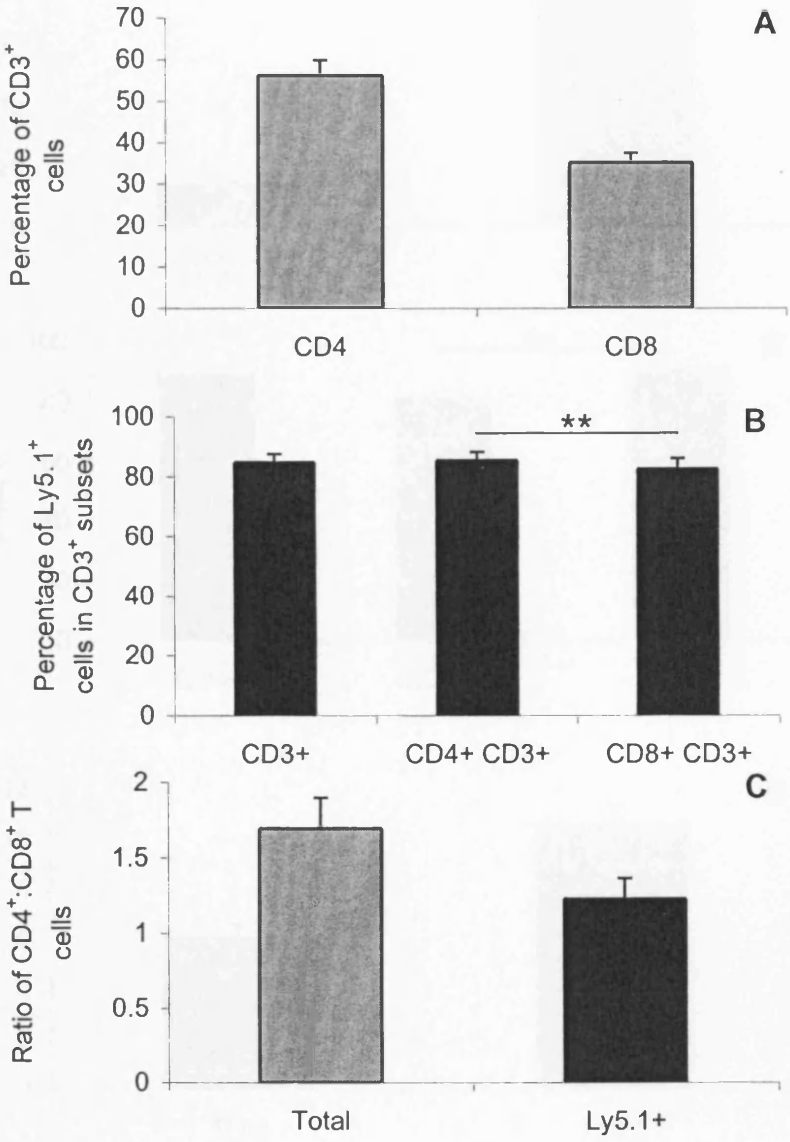

Figure 3.31. Analysis of CD4 and CD8 T cells in lymph nodes of $CD44^{+/+}$ (Ly5.1) → $CD44^{+/+}$ (Ly5.2) chimeras.

A. Percentages of $CD4^+$ and $CD8^+$ $CD3^+$ cells. B. The percentage of $Ly5.1^+$ cells in each population. The CD4:CD8 T cell ratio among total and donor cells. Data are mean ± s.e.m. obtained from 2 groups of 4 mice. P values, 2-sample t test; **, $P<0.01$

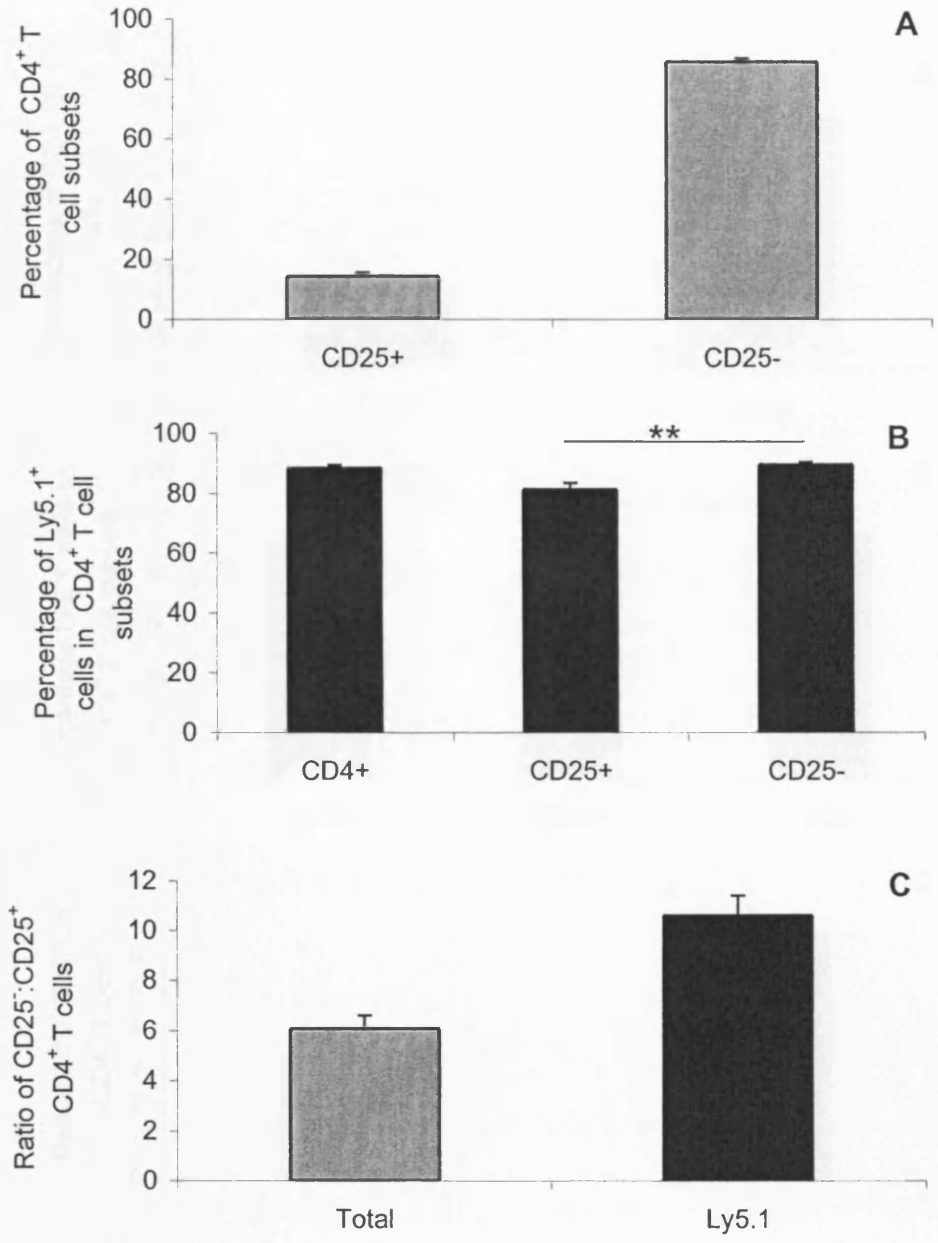

Figure 3.32. Analysis of CD4 subsets in spleens of $CD44^{+/+}$ (Ly5.1) → $CD44^{+/+}$ (Ly5.2) chimeras.

A. Percentages of $CD25^+$ and $CD25^-$ $CD4^+$ $CD3^+$ cells in spleen. B. The percentage of $Ly5.1^+$ cells in each CD4 subset. C. The $CD25^-$ $CD4^+$:$CD25^+$ $CD4^+$ T cell ratio among total and donor ($Ly5.1^+$) cells. Data are mean ± s.e.m. obtained from 1 group of 4 mice. P values, 2-sample t test; **, $P<0.01$

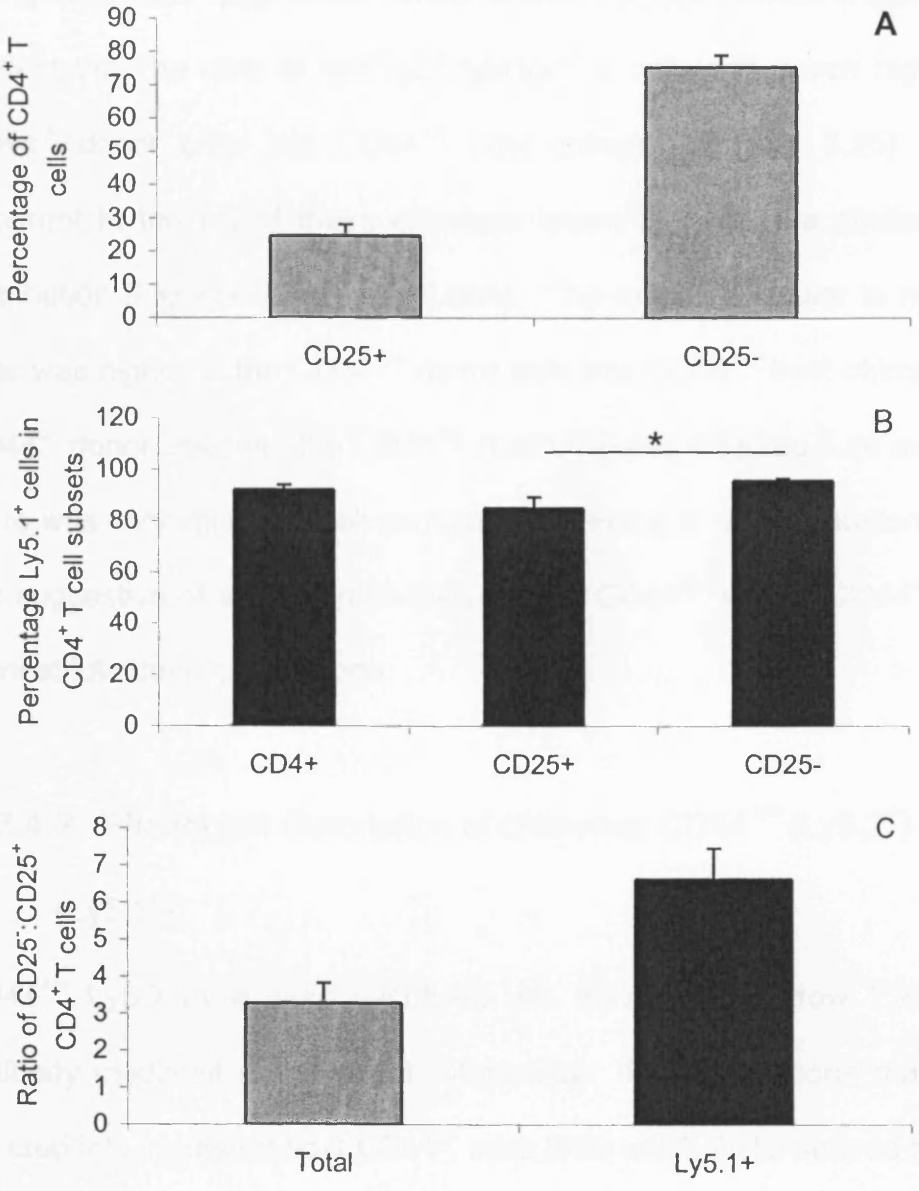

Figure 3.33. Analysis of CD4 subsets in lymph nodes of $CD44^{+/+}$ (Ly5.1) → $CD44^{+/+}$ (Ly5.2) chimeras.

A. Percentages of $CD25^+$ and $CD25^-$ $CD4^+$ $CD3^+$ cells in lymph nodes. B. The percentage of $Ly5.1^+$ cells in each CD4 subset. C. The $CD25^-$ $CD4^+$:$CD25^+$ $CD4^+$ T cell ratio among total and donor ($Ly5.1^+$) cells. Data are mean ± s.e.m. obtained from 1 group of 4 mice. P values, 2-sample t test; *, $P<0.05$

Although the distribution of B cell subsets defined by sIgM and sIgD expression in the splenic $B220^+$ population varied between groups of mice (Figure 3.34A) it was evident that the ratio of IgM^+IgD^+:IgM^-IgD^+ B cells was much higher than in the $CD44^{-/-}$ donor cells into $CD44^{+/+}$ host chimeras (Figure 3.25) This was not apparent in the LN of these chimeras where there was a similar B cell subset distribution (Figures 3.35A and 3.26A). The ratio of follicular to marginal zone B cells was higher in the $CD44^{+/+}$ donor cells into $CD44^{+/+}$ host chimeras than in the $CD44^{-/-}$ donor cells into the $CD44^{+/+}$ host chimeras (Figures 3.36 and 3.27). Since there was very little host cell contamination in the B cell populations, these results are suggestive of a differential ability of the $CD44^{+/+}$ versus $CD44^{-/-}$ progenitors to reconstitute these populations.

3.3.4.2. Phenotypic description of chimeras: $CD44^{+/+}$ ($Ly5.2^+$) → $CD44^{-/-}$ ($Ly5.1^+$).

$CD44^{+/+}$ Ly5.2 mice were sacrificed and their bone marrow T cell depleted by antibody mediated complement cytotoxicity. T-depleted bone marrow cells were injected into irradiated host $CD44^{-/-}$ mice (900 rads). Cells derived from the $CD44^{-/-}$ the host cells were identified in the reconstituted chimeras using an anti-Ly5.1 (CD45.2) antibody, while donor-derived cells were detected with an anti-Ly5.2 (CD45.1) antibody.

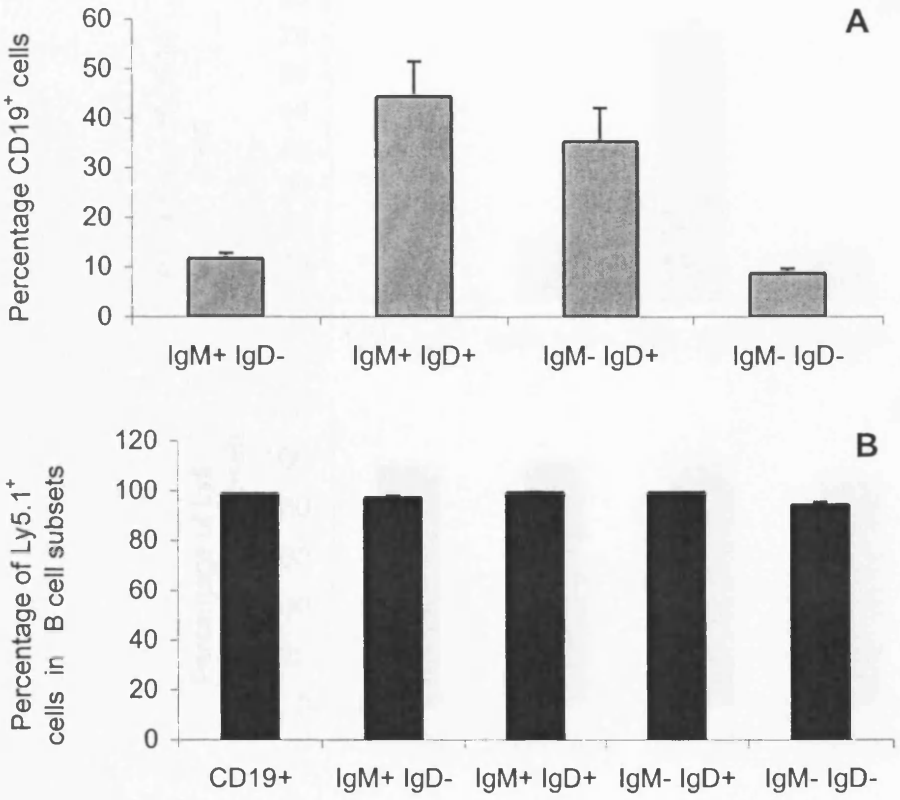

Figure 3.34. Analysis of B cell subsets in spleens of $CD44^{+/+}$ (Ly5.1) → $CD44^{+/+}$ (Ly5.2) chimeras.

A. Percentages of splenic B cell ($CD19^+$) subsets defined by sIgM and sIgD expression. B. The percentage of $Ly5.1^+$ cells in each B cell subset. Data are mean ± s.e.m.obtained from 2 groups of 4 mice.

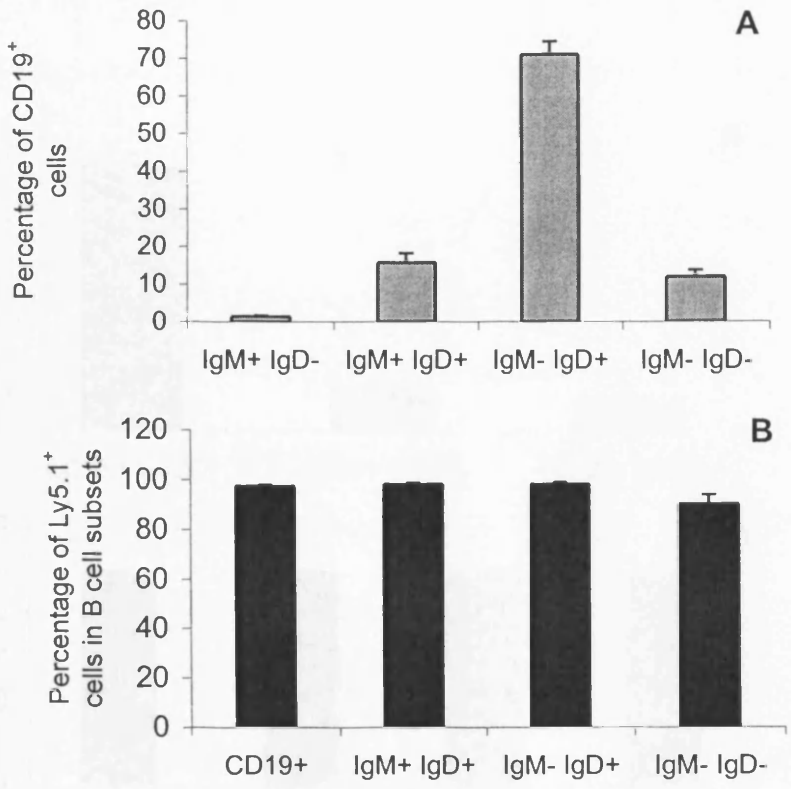

Figure 3.35. Analysis of B cell subsets in lymph nodes of $CD44^{+/+}$ (Ly5.1) → $CD44^{+/+}$ (Ly5.2) chimeras.

A. Percentages of lymph node B cell ($CD19^+$) subsets defined by sIgM and sIgD expression. B. The percentage of $Ly5.1^+$ cells in each B cell subset. Data are mean ± s.e.m. obtained from 2 groups of 4 mice.

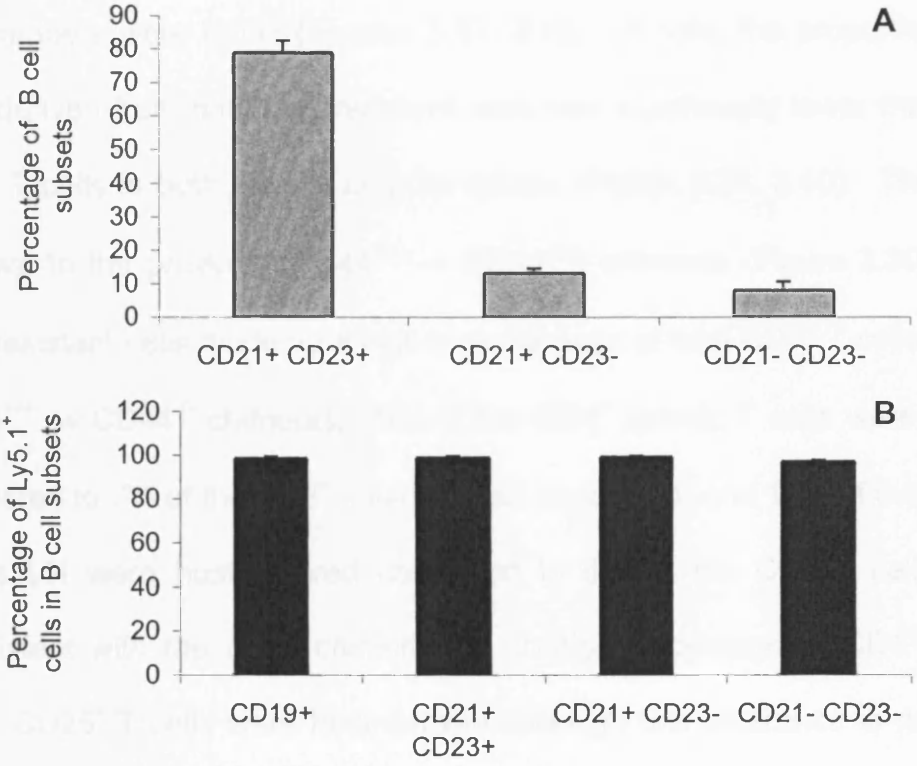

Figure 3.36. Analysis of follicular and marginal zone B cells in spleen of $CD44^{+/+}$ (Ly5.1) → $CD44^{+/+}$ (Ly5.2) chimeras.

A. Percentages of splenic B cell ($CD19^{+}$) subsets defined by surface expression of CD21 and CD23. B. The percentage of $Ly5.1^{+}$ cells in each B cell subset. Data are mean ± s.e.m. obtained from 2 groups of 4 mice.

As in the other chimeras, there was a significant proportion (15%) of T cells that represented radioresistant $CD44^{-/-}$ host cells. Of host B cells, conversely only very low numbers were found (Figures 3.37, 3.48). Of note, the proportion of $CD8^+$ T cells derived from host radioresistant cells was significantly lower than that of the $CD4^+$ T cells in both the LN and the spleen (Figure 3.39, 3.40). This is in direct contrast to the previous ($CD44^{+/+} \rightarrow CD44^{+/+}$) chimeras (Figure 3.30) where host radioresistant cells made up a higher percentage of total $CD8^+$ T cells in the LN. In $CD44^{+/+} \rightarrow CD44^{-/-}$ chimeras, 19% of the $CD4^+$ splenic T cells were host derived compared to 7% of the $CD8^+$ splenic T cell population, and 15% of the $CD4^+$ T cells in the LN were host derived compared to 5% of the $CD8^+$T cells in the LN. Consistent with the other chimeras, a greater percentage of $CD4^+$ $CD25^+$ than $CD4^+$ $CD25^-$ T cells were host derived although this difference is not statistically significant (Figures 3.41, 3.42).

The distribution of B cell subsets defined by sIgM and sIgD expression in the splenic $B220^+$ population was constant between mice (Figure 3.43A, 3.44A) with a similar proportion of radioresistant host $CD44^{-/-}$ B cells (approximately 1.5) (Figure 3.43B). However, there did appear to be a slightly higher proportion of host $sIgM^-$ $sIgD^-$ B cells in the LN.

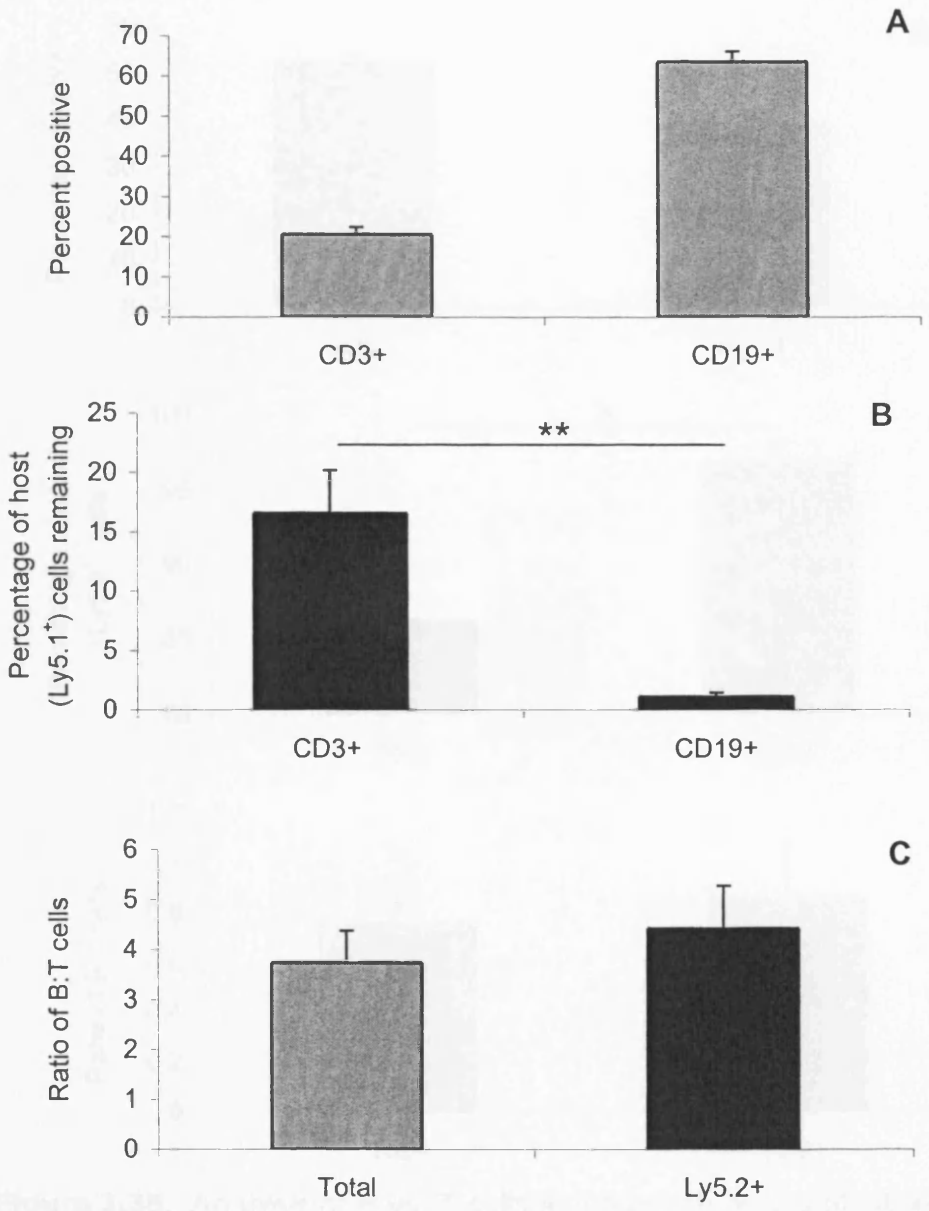

Figure 3.37. Analysis of B versus T cells in the spleen of $CD44^{+/+}$ (Ly5.2)→ $CD44^{-/-}$ (Ly5.1) chimeras.

A. Percentages of T and B cells in the spleen. B. The percentage of host $Ly5.1^{+}$ cells present in T and B cell splenic populations. C. B:T cell ratio among total and donor cells. Data are mean ± s.e.m. obtained from 1 group of 6 mice. P values, 2 sample t test; **, $P<0.01$

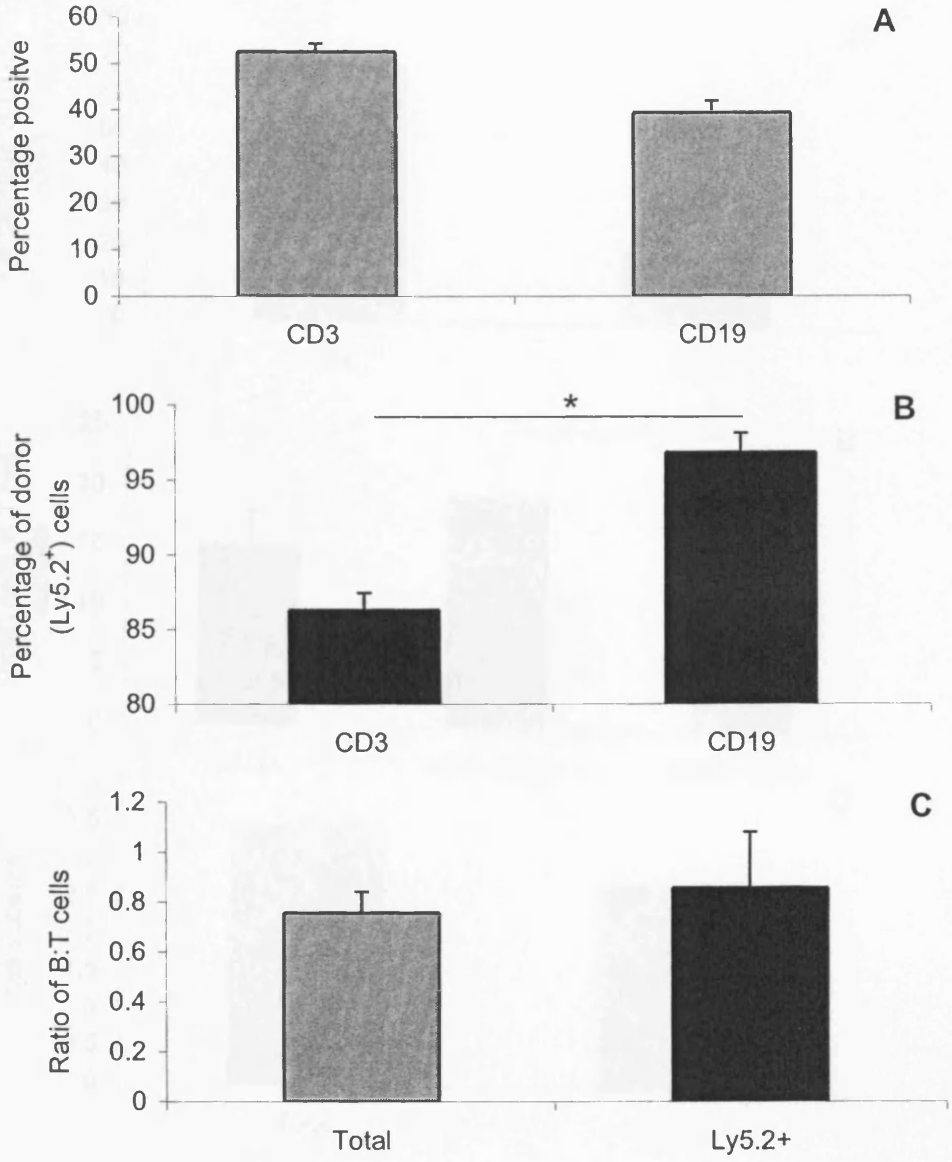

Figure 3.38. Analysis of B vs. T cells in the lymph nodes of $CD44^{+/+}$ (Ly5.2)→ $CD44^{-/-}$ (Ly5.1) chimeras.

A. Percentages of T and B cells in the lymph nodes. B. The percentage of donor $Ly5.2^{+}$ cells present in T and B cell populations. C. B:T cell ratio among total and donor cells. Data are mean ± s.e.m. obtained from 1 group of 6 mice. P values, 2-sample t test; **, $P<0.01$

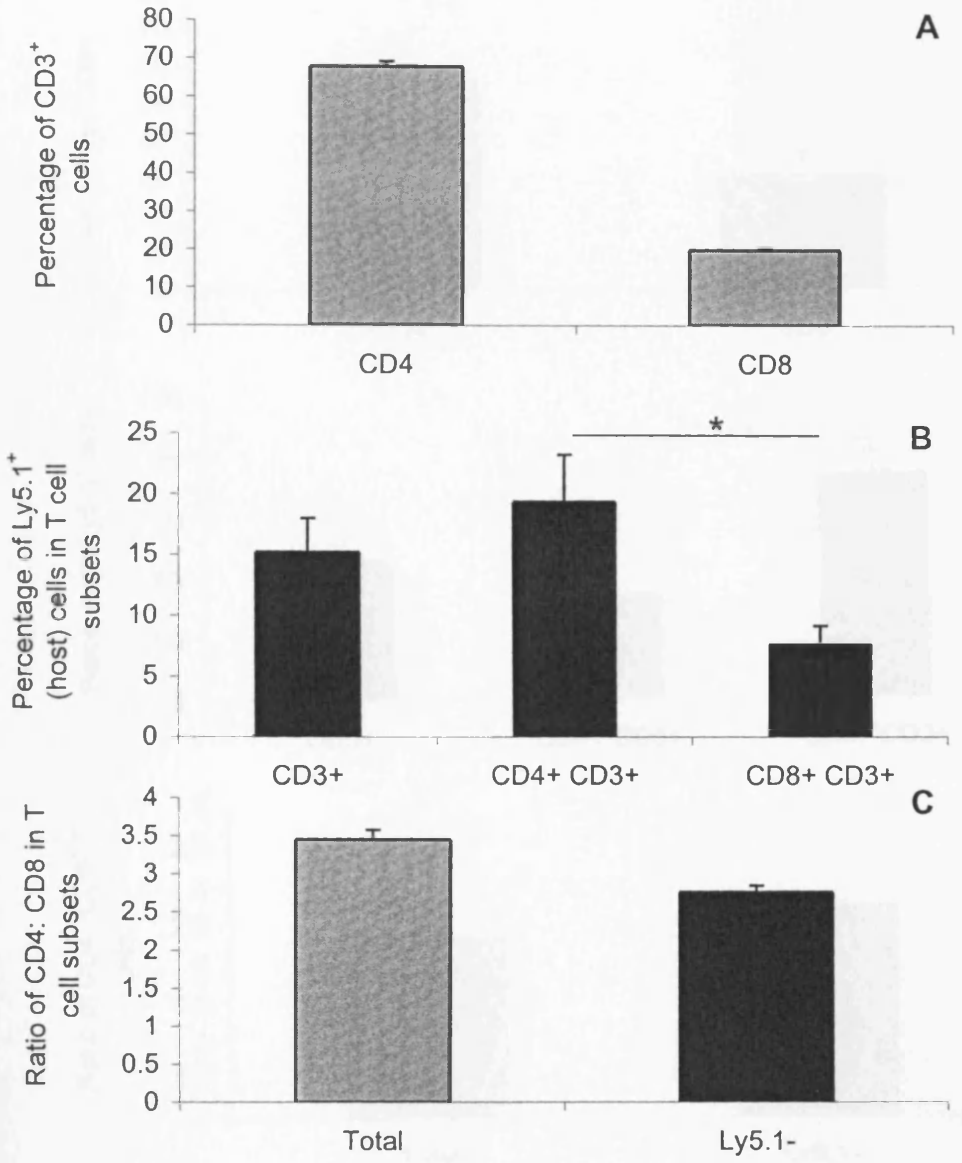

Figure 3.39. Analysis of CD4 and CD8 T cells in spleens of $CD44^{+/+}$ (Ly5.2) → $CD44^{-/-}$ (Ly5.1) chimeras.

A. Percentages of $CD4^+$ and $CD8^+$ $CD3^+$ cells in spleen. B. Percentage of host $Ly5.1^+$ cells in each splenic population. C. The CD4:CD8 T cell ratio among total and donor ($Ly5.1^-$) cells. Data are mean ± s.e.m. obtained from 1 group of 6 mice. P values, 2-sample t test; *, $P<0.05$

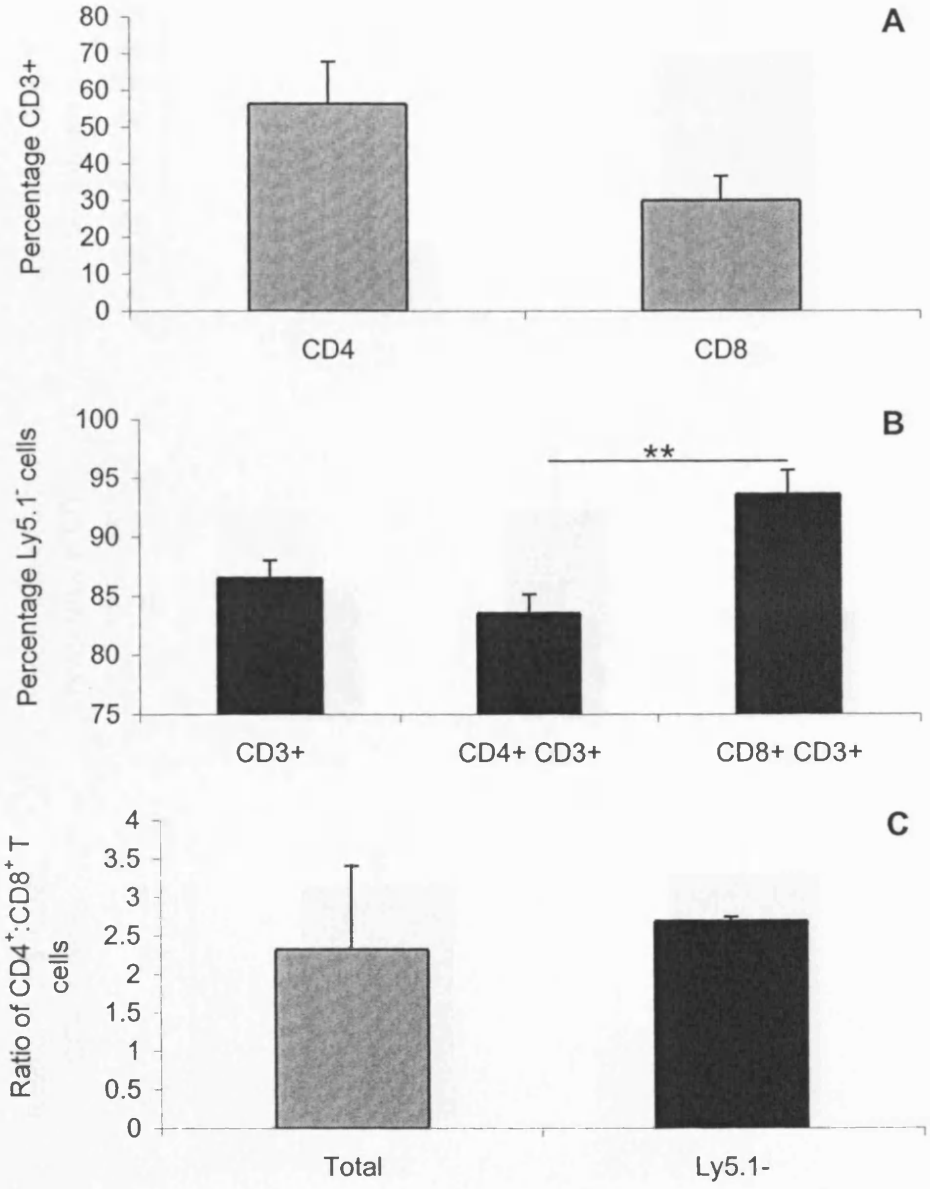

Figure 3.40. Analysis of CD4 and CD8 T cells in lymph nodes of $CD44^{+/+}$ (Ly5.2) → $CD44^{-/-}$ (Ly5.1) chimeras.

A. Percentages of $CD4^+$ and $CD8^+$ $CD3^+$ cells in lymph nodes. B. Percentage of donor $Ly5.1^-$ cells in each population. C. The CD4:CD8 T cell ratio among total and donor ($Ly5.1^-$) cells. Data are mean ± s.e.m. obtained from 1 group of 6 mice. P values, 2-sample t test; **, $P<0.01$

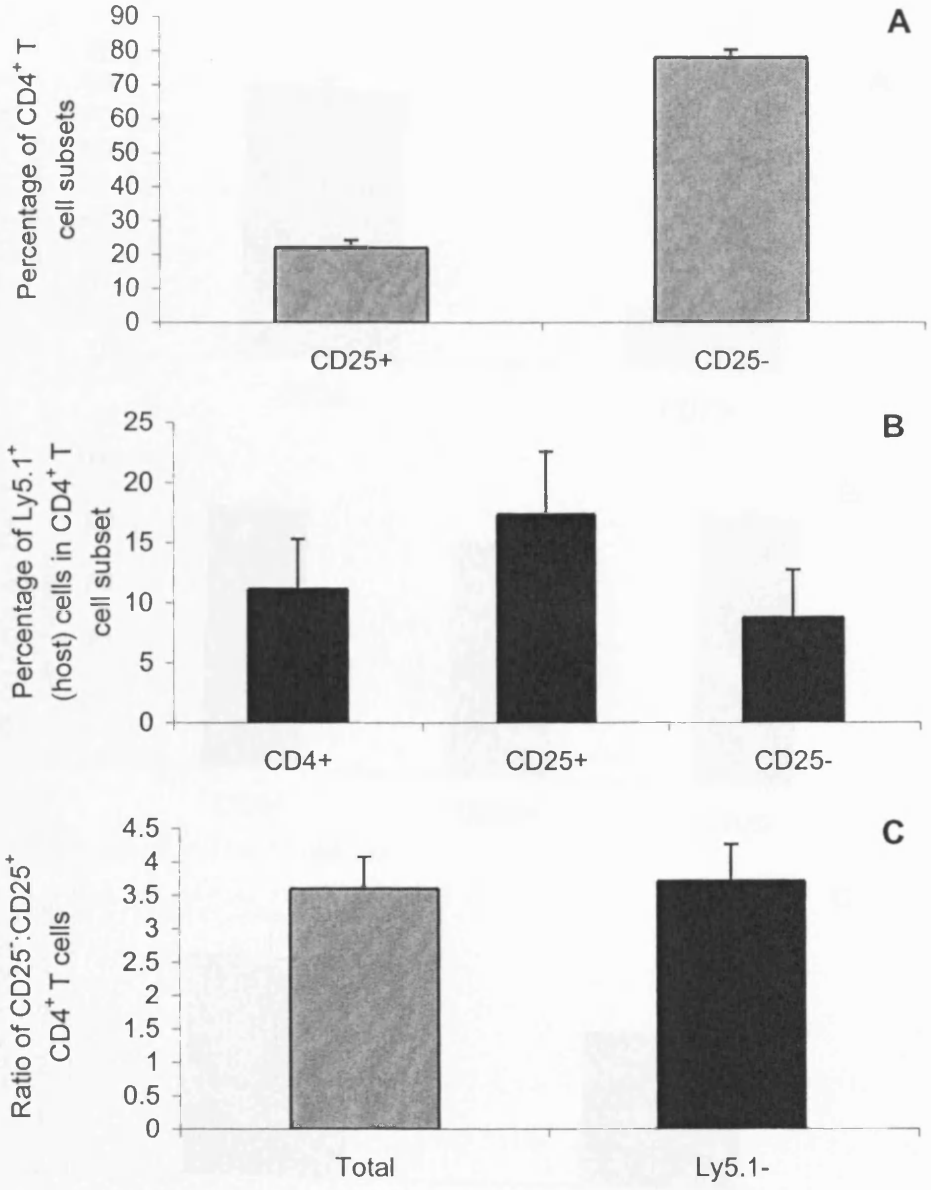

Figure 3.41. Analysis of CD4 subsets in spleens of $CD44^{+/+}$ (Ly5.2) → $CD44^{-/-}$ (Ly5.1) chimeras.

A. Percentages of $CD25^+$ and $CD25^-$ $CD4^+$ $CD3^+$ cells in spleen. B. The percentage of $Ly5.1^+$ (host) cells remaining in each CD4 subset. C. The $CD25^-CD4^+$:$CD25^+$ $CD4^+$ T cell ratio among total and donor ($Ly5.1^-$) cells. Data are mean ± s.e.m. obtained from 1 group of 2 mice.

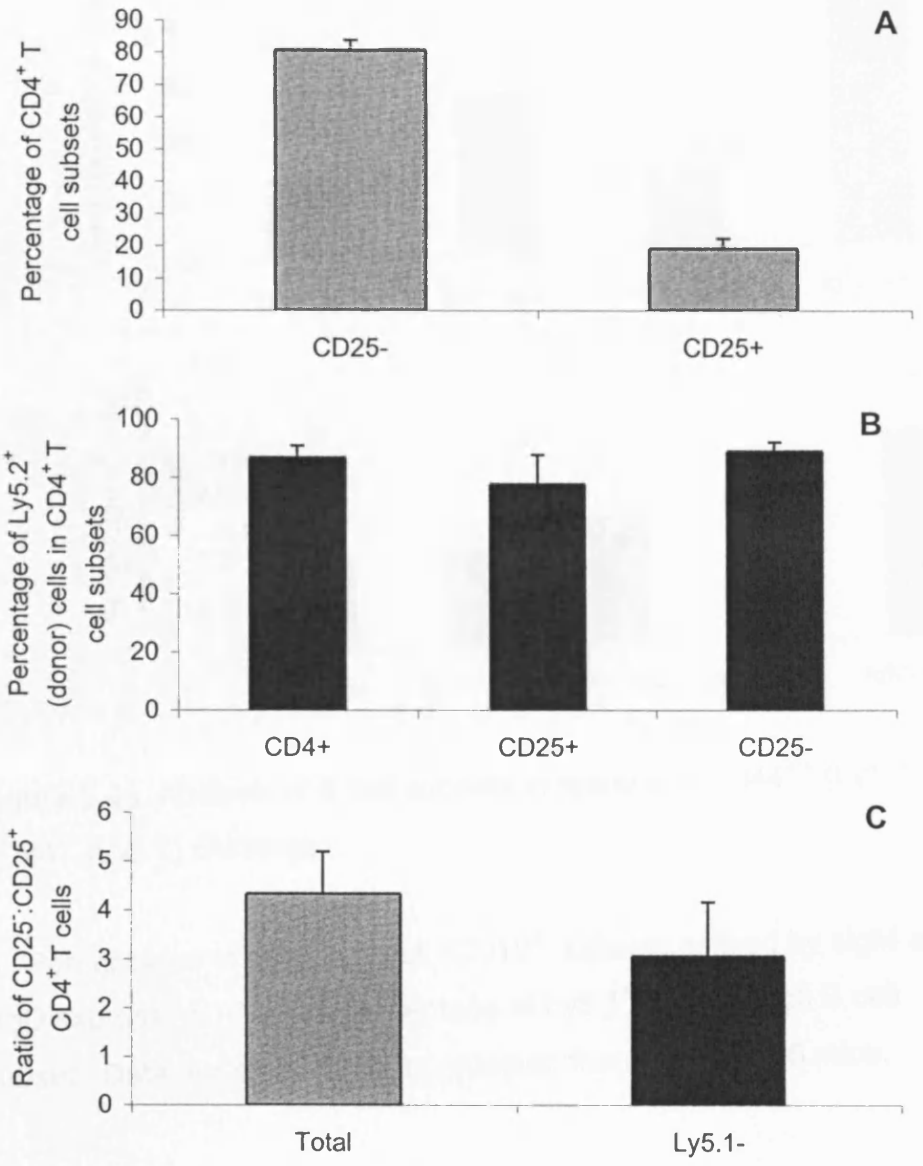

Figure 3.42. Analysis of CD4 subsets in lymph nodes of $CD44^{+/+}$ (Ly5.2) → $CD44^{-/-}$ (Ly5.1) chimeras.

A. Percentages of $CD25^+$ and $CD25^-$ $CD4^+$ $CD3^+$ cells in lymph nodes. B. The percentage of $Ly5.2^+$ (host) cells in each CD4 subset. C. The $CD25^-CD4^+$:$CD25^+CD4^+$ T cell ratio among total and donor ($Ly5.1^-$) cells. Data are mean ± s.e.m. obtained from 1 group of 2 mice.

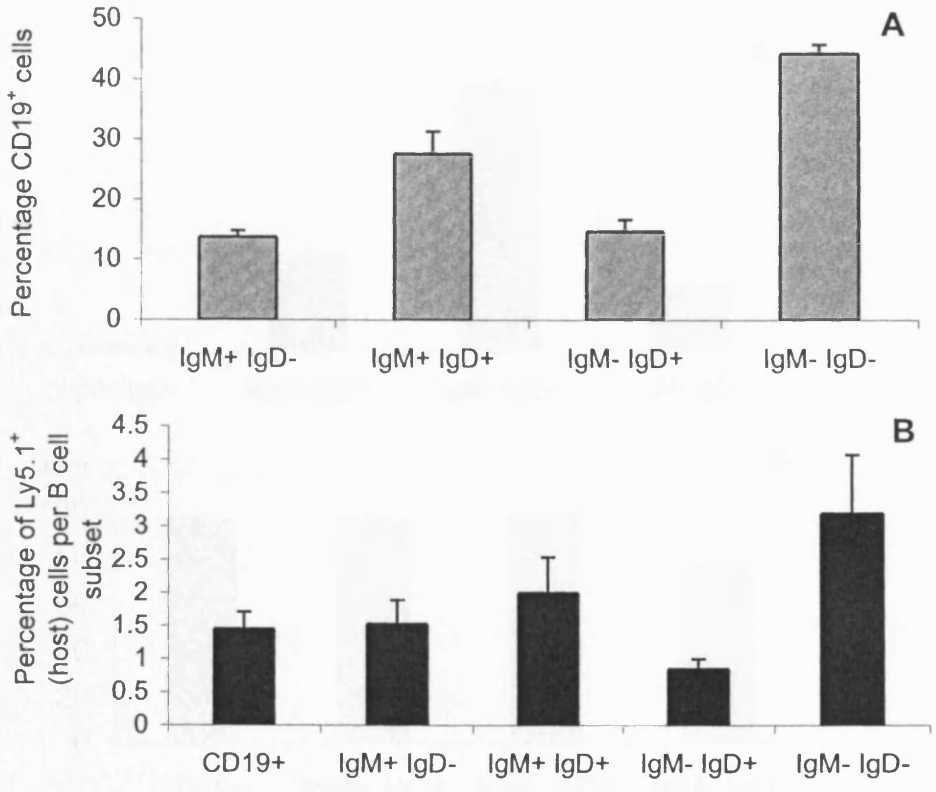

Figure 3.43. Analysis of B cell subsets in spleens of $CD44^{+/+}$ (Ly5.1) → $CD44^{-/-}$ (Ly5.1) chimeras.

A. Percentages of splenic B cell ($CD19^+$) subsets defined by sIgM and sIgD expression. B. The percentage of $Ly5.1^+$ cells in each B cell subset. Data are mean ± s.e.m. obtained from 1 group of 6 mice.

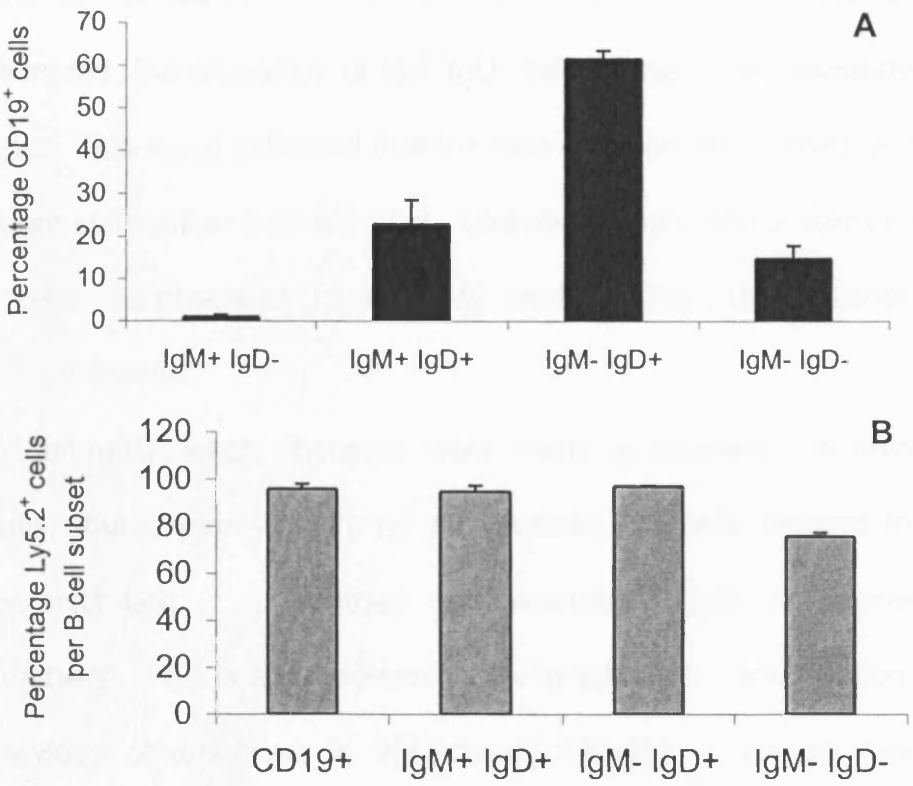

Figure 3.44. Analysis of B cell subsets in lymph nodes of $CD44^{+/+}$ (Ly5.2) → $CD44^{-/-}$ (Ly5.1) chimeras.

A. Percentages of B cell ($CD19^{+}$) subsets defined by sIgM and sIgD expression in lymph nodes. B. The percentage of $Ly5.2^{+}$ (donor) cells in each B cell subset. Data are mean ± s.e.m. obtained from 1 group of 2 mice.

Like in the $CD44^{+/+} \rightarrow CD44^{+/+}$ chimeras, but unlike the $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras, the proportion of $IgM^{+}IgD^{+}$ cells in the spleen exceeded that of $IgM^{-}IgD^{+}$ cells. This result indicated that the ratio of these cell subsets was a property of the donor cells rather than the host. Likewise a high ratio of follicular to marginal zone B cells was observed (Figure 3.45), similar to the $CD44^{+/+}$ donor cells into $CD44^{+/+}$ host chimeras.

In summary, when chimeras were made to examine the level of radioresistant cells, there were virtually no radioresistant B cells present in these mice after reconstitution. In contrast radioresistant T cells were present in significant numbers. This is an important factor to take into consideration with regard to the previous observations in the mixed chimeras. Hence, host derived T cells complicate the interpretation of a deficient ability of the $CD44^{-/-}$ progenitors to produce T cells in these chimeras. Of note there were fewer radioresistant cells found in the LN of these mice, suggesting that the composition of lymph node cells present in these mice are more representative of donor derived cells, with less of an influence from host radioresistant cells.

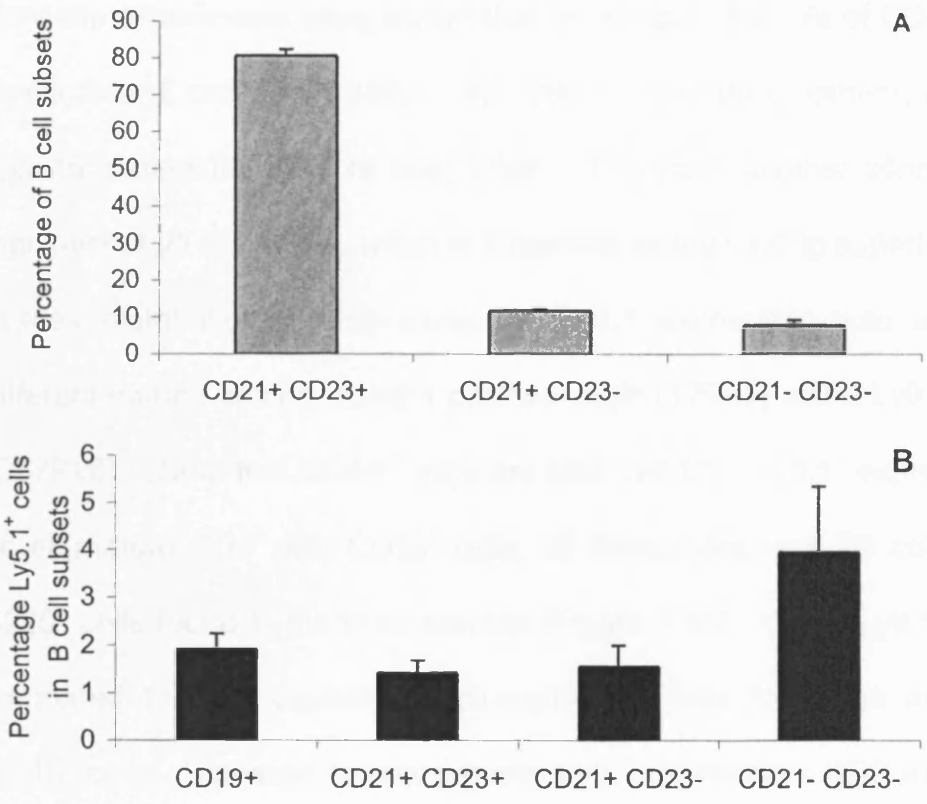

Figure 3.45. Analysis of follicular and marginal zone B cells in spleens of $CD44^{+/+}$ (Ly5.2) → $CD44^{-/-}$ (Ly5.1) chimeras.

A. Percentages of splenic B cell ($CD19^{+}$) subsets defined by CD21 and CD23 expression. B. The percentage of $Ly5.1^{+}$ (host) cells in each B cell subset. Data are mean ± s.e.m. obtained from 1 group of 6 mice.

3.3.5. CD229

Previous experiments have shown that to elucidate the role of CD44 in lymphocyte development, most especially in the role in T cell development, it is important be able to distinguish cells of host origin. Therefore another allotypic marker was employed: Ly9 or CD229, which is a member of the CD2 Ig superfamily. Initially, to assess distribution of this molecule, Ly9.1 expression was examined in two different murine strains, a Ly9.1 positive strain (129Sv) and a Ly9.1 negative strain (C57Bl/6). (Note that $CD44^{-/-}$ mice are also $Ly9.1^{+}$). $Ly9.1^{+}$ expression was found on all mature $CD3^{+}$ and $CD19^{+}$ cells, all thymocytes, and B1 cells, as well as all $B220^{+}$ cells found in the bone marrow (Figure 3.46). When Ly9.1 expression was compared to CD2 expression, co-expression was found on mature $CD3^{+}$ and $CD19^{+}$ cells. However, in contrast with Ly9.1 expression, CD2 was not expressed on all immature B or T cells found in the bone marrow and thymus (Figure 3.47). Overall, therefore, these results showed that Ly9.1 could serve as a useful allotypic marker to detect T and B cells at all stages of maturation.

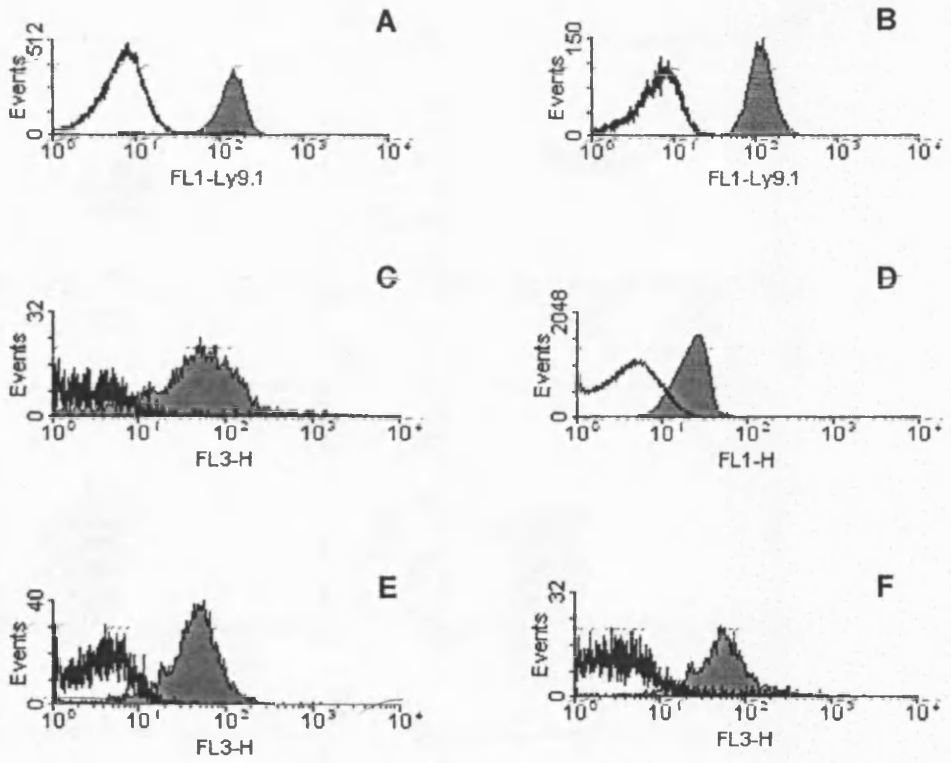

Figure 3.46. Ly9.1 expression in various cell types

A. Ly9.1$^+$ expression in splenic CD3$^+$ cells. B. Ly9.1 expression in splenic CD19$^+$ cells. C. Ly9.1 expression in CD11b$^+$ B220$^+$ peritoneal B cells. D. Ly9.1 expression in total thymocytes. E. Ly9.1 expression in B220$^+$ sIgM$^-$ bone marrow cells. F. Ly9.1 expression in B220$^+$ IgM$^+$ bone marow cells. Data are representative of results from 2 mice. ▩ 129 mice, ▬ B6 mice

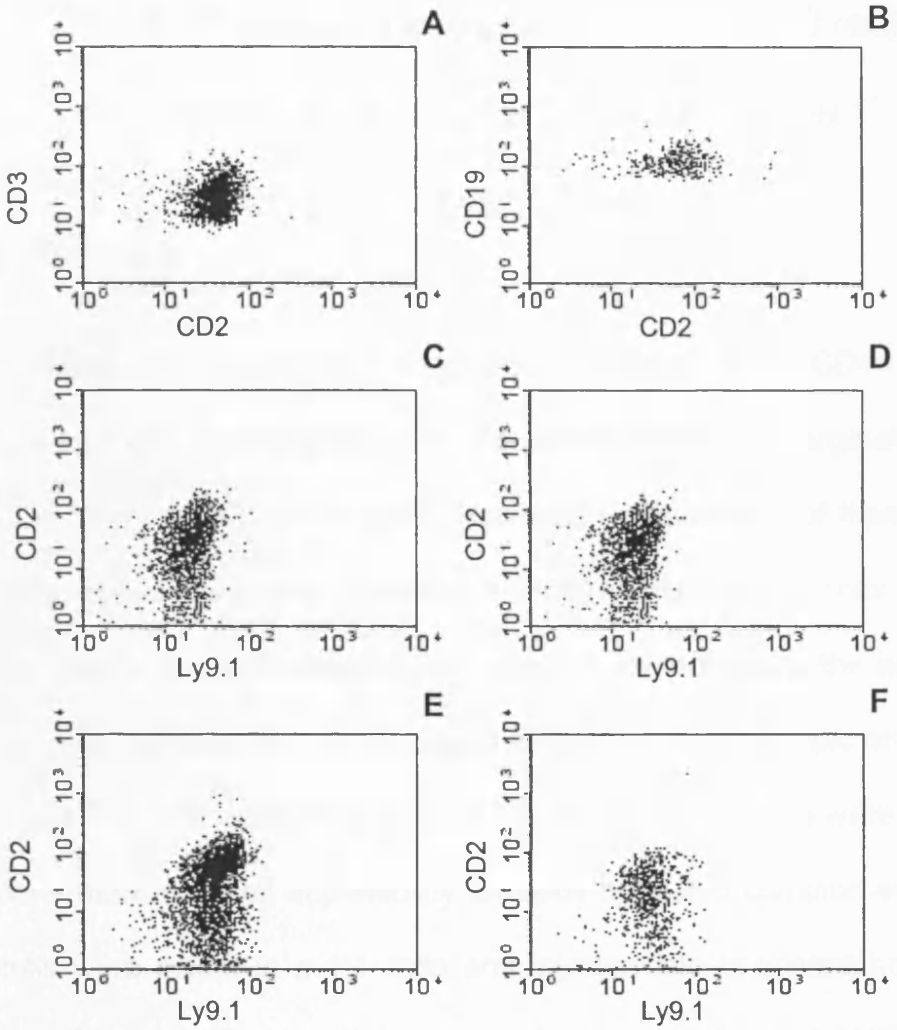

Figure 3.47. CD2 expression in various cell types.

A. CD2 expression in splenic $CD3^+$ cells. B. CD2 expression in splenic $CD19^+$ cells. C. CD2 vs. Ly9.1 expression in total thymocytes. D. CD2 vs. Ly9.1 expression in double negative thymocytes. E. CD2 vs. Ly9.1 expression in $B220^+$ bone marrow cells. F. CD2 vs. Ly9.1 expression in $B220^+$ $sIgM^-$ bone marrow cells. Data are representative of results from 2 129 mice.

3.3.6. Phenotypic description of mixed (1100 rad) chimeras: $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) and $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.1^{+}$) hosts

Previous experiments have suggested a putative role for CD44 in B cell and T cell development, and especially in the development of marginal zone B cells and $CD4^{+}$ and $CD25^{+}CD4^{+}$ T cells. However, interpretation of these experiments was complicated due to the presence of radioresistant host T cells. This difficulty was resolved in this experiment in two ways: A, by increasing the amount or irradiation given to the host mice, B, by using Ly9.1 as another allotypic marker.

$CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) and $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) mice were sacrificed and their bone marrow T cell depleted by antibody mediated complement cytotoxicity. The cells were mixed in a 1:1 ratio and injected into irradiated host $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.1^{+}$) mice. The host mice were given a split dose of 1100 rads, i.e. 2 X 550 rads, 5 hours apart. Once immune reconstitution had occurred, the mice were sacrificed, and their phenotype was analysed by FACS. Cells derived from the $CD44^{-/-}$ progenitor cells were identified using an anti-Ly9.1 (CD229.1) antibody, and cells derived from $CD44^{+/+}$ donor bone marrow was identified using an anti-Ly5.2 (CD45.1) antibody. B cells were identified using the anti-Ly9.1 antibody alone, all other B cells were assumed to be $CD44^{+/+}$ donor cells due to lack of radioresistant B cells. Donor T cells were identified using the anti-Ly9.1 antibody and the anti-Ly5.2 antibody. Occasionally, due to requirements for specific fluorochrome-antibody combinations, an anti-$Ly5.1^{+}$ antibody was used with an anti $Ly9.1^{+}$ antibody, in which case $Ly5.1^{-}$ lymphocytes were considered to be $Ly5.2^{+}$

$CD44^{+/+}$ donor cells and $Ly5.1^{+}$ $Ly9.1^{+}$ cells were determined to be $Ly5.1^{+}$ $CD44^{-/-}$ donor cells.

These mice had normal cell numbers in their spleens and LN (appendix 1), and normal percentages of total T and B cells, (Figures 3.48A, 3.49A). However when the T and B cells from these chimeras were analysed there was again a difference in the origin of these cells (Figures 3.48B, 3.49B). It appeared that $CD44^{-/-}$ progenitors still preferentially develop into B cells whereas the $CD44^{+/+}$ cells develop more efficiently into T cells. Although the percentages of donor $CD44^{-/-}$ cells found in these chimeras were less than those found in our previous set of mixed chimeras, the basic findings were consistent with what was observed before. The B:T cell ratio among the $CD44^{-/-}$ cells was calculated and compared to that of total and donor ($Ly5.2^{+}$) $CD44^{+/+}$ cells (Figures 3.48C, 3.49C). In the spleen there were 3.5 $CD44^{-/-}$ B cells to every $CD44^{-/-}$ T cell, compared to 2 B cells to every T cell among $CD44^{+/+}$ cells (similar for total or donor $CD44^{+/+}$ cells). In the LN there was a similar pattern, with 1.2 $CD44^{-/-}$ B cells per $CD44^{-/-}$ T cell compared to 0.7 B cell to every T cell for $CD44^{+/+}$ cells.

In the spleen and LN, again there was a significant difference in the ability of $CD44^{-/-}$ progenitors to develop into either $CD4^{+}$ or $CD8^{+}$ T cells (Figures 3.50, 3.51).

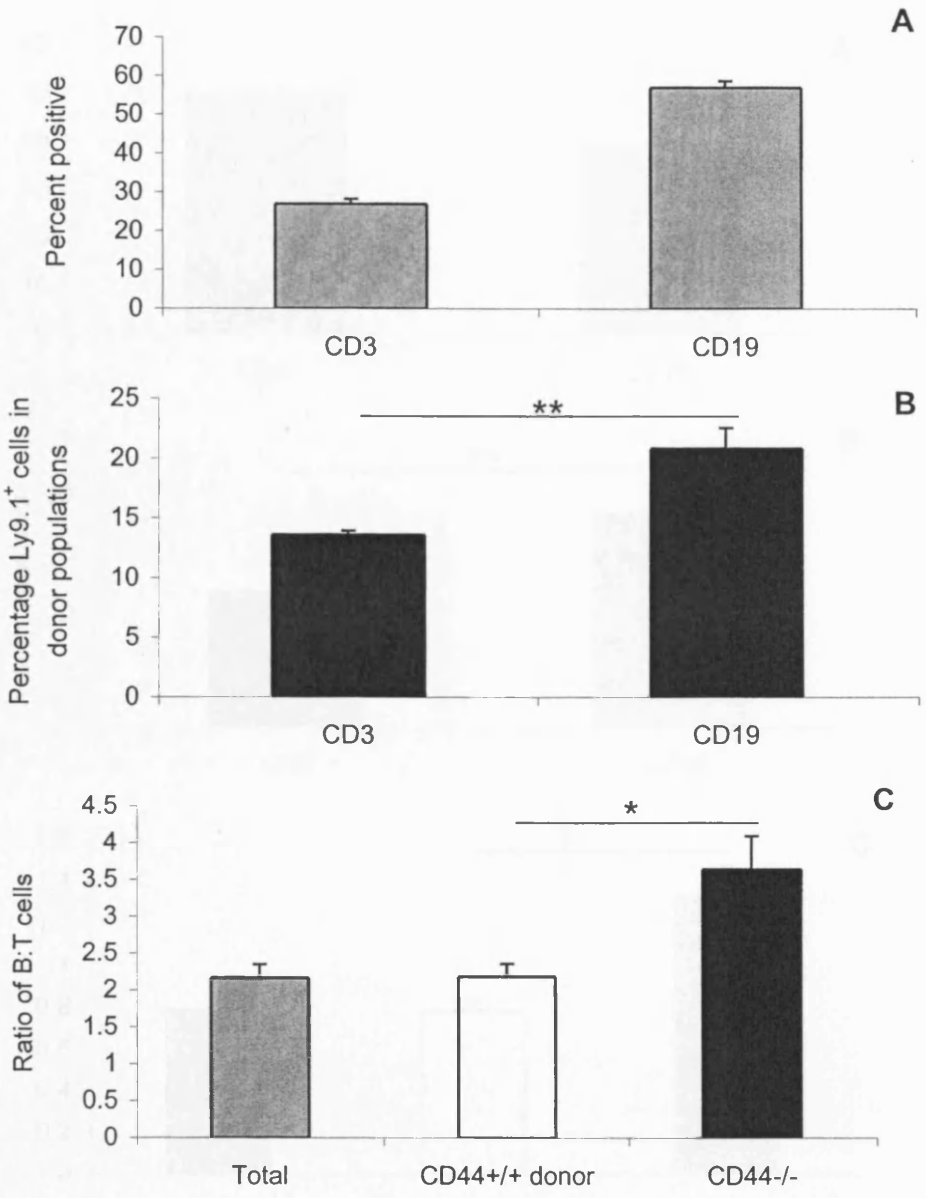

Figure 3.48. Analysis of T and B cells in $CD44^{+/+}$ & $CD44^{-/-}$→ $CD44^{+/+}$ (1100 rads) chimeras.

A. Percentages of T and B cells in spleen. B. The percentage of $Ly9.1^{+}$ cells in each splenic population. C. The B:T cell ratio among total and $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean ± s.e.m. obtained from 2 groups of chimeras: 1 group of 2 and one group of 3. P values, 2-sample t test ; *, $P<0.05$, **, $P<0.01$

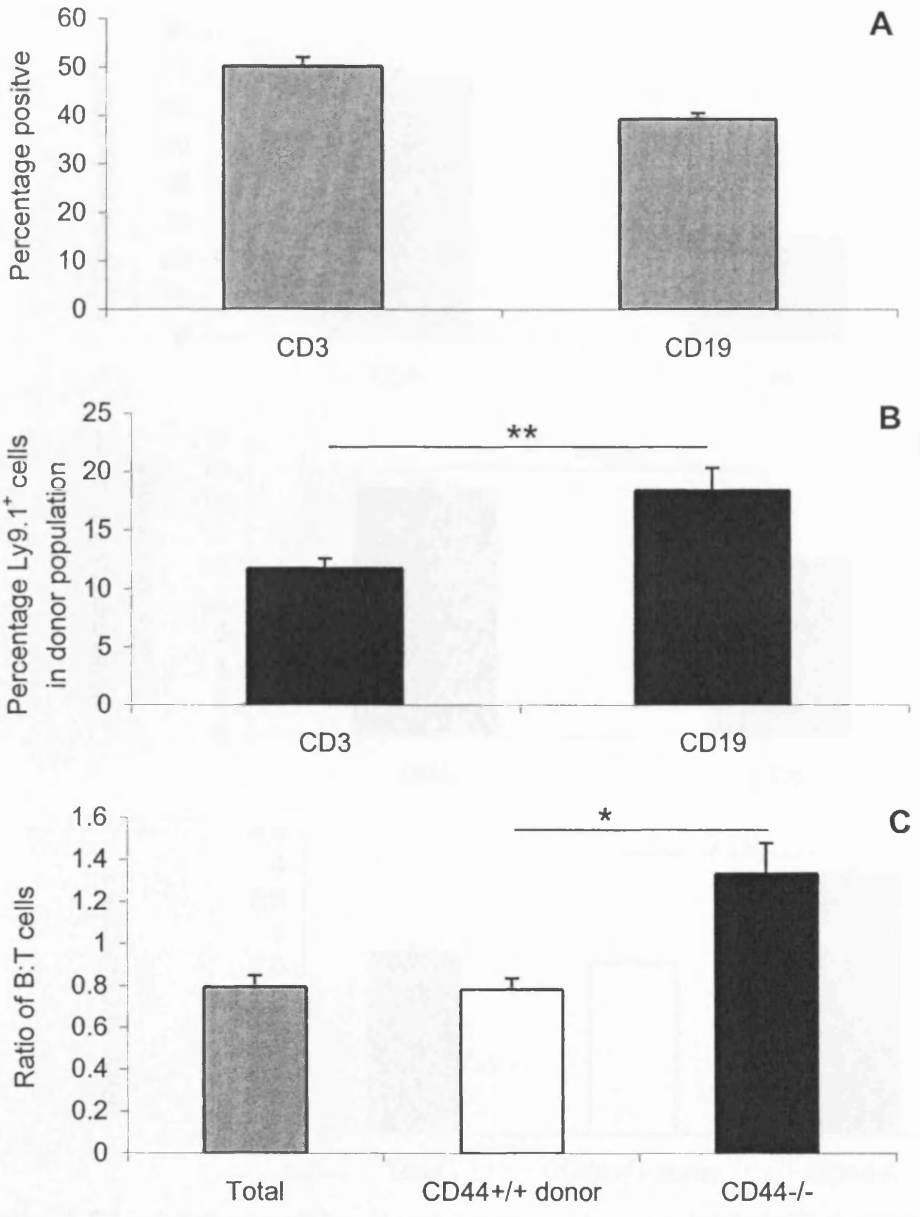

Figure 3.49. Analysis of T and B cells in lymph nodes of $CD44^{+/+}$ & $CD44^{-/-}\rightarrow CD44^{+/+}$ (1100 rads) chimeras.

A. Percentages of T and B cells in lymph nodes. B. The percentage of $Ly9.1^{+}$ cells in each population. C. The B:T cell ratio among total and $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean ± s.e.m. obtained from 2 groups of chimeras: 1 group of 2 and one group of 3. P values, 2-sample t test ; *, $P<0.05$, **, $P<0.01$

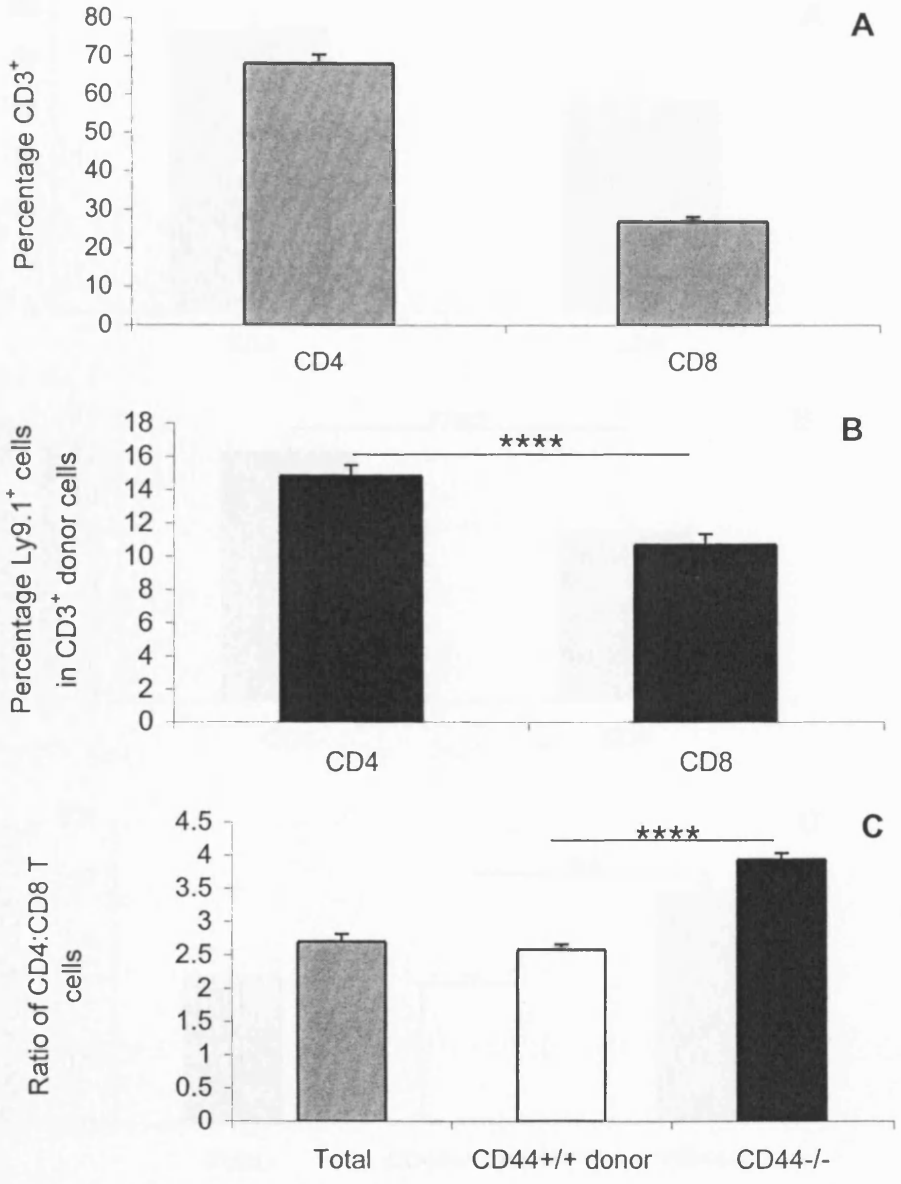

Figure 3.50. Analysis of T cell subsets in spleens of $CD44^{+/+}$ & $CD44^{-/-}$ → $CD44^{+/+}$ (1100rads) chimeras.

A. Percentages of $CD4^+$ and $CD8^+$ T cells in spleens. B. The percentage of $Ly9.1^+$ cells in each donor T cell population. C. The B:T cell ratio among total and $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 groups of 3. P values, 2-sample t test; ****, $P<0.001$

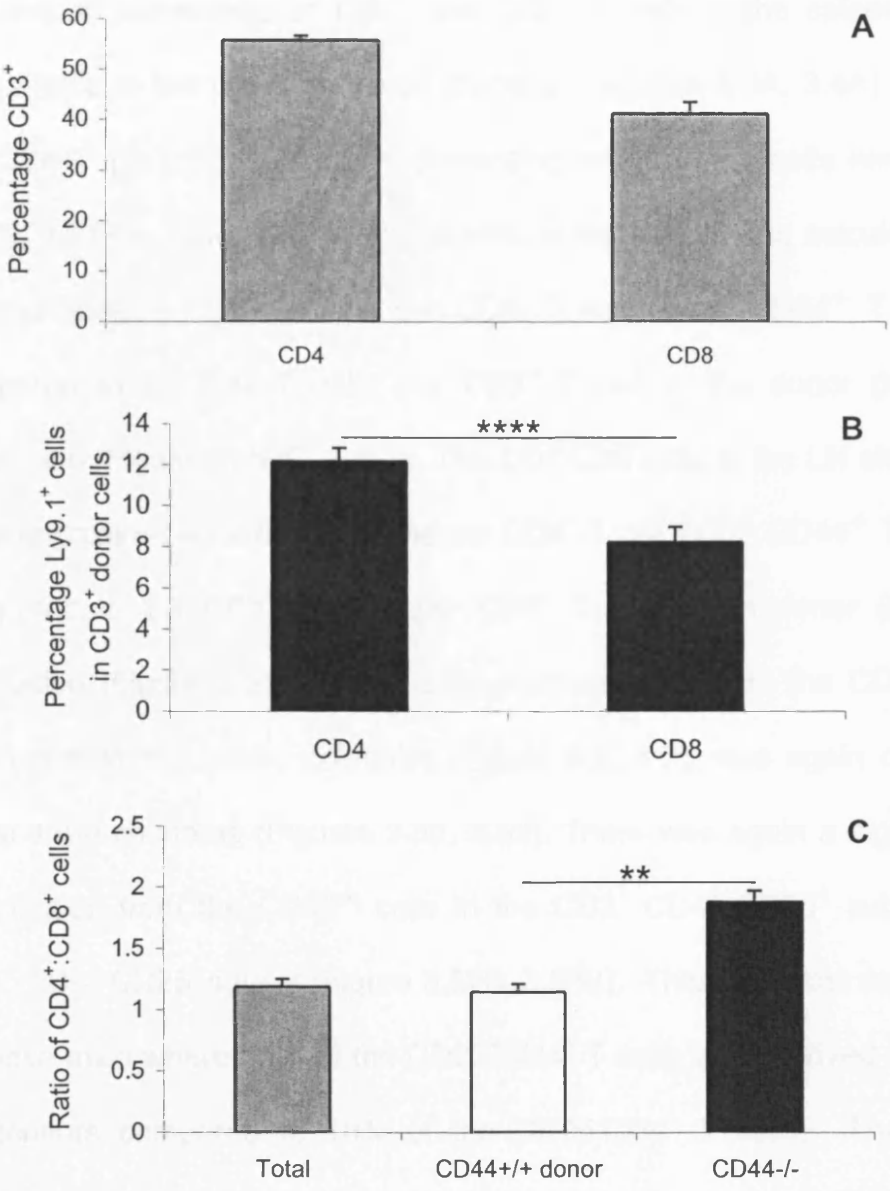

Figure 3.51. Analysis of T cell subsets in lymph nodes of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ (1100 rads) chimeras.

A. Percentages of $CD4^+$ and $CD8^+$ T cells in lymph nodes. B. The percentage of $Ly9.1^+$ cells in each donor T cell population. C. The B:T cell ratio among total and $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 2 group of 2 and 1 group of 3. P values, 2-sample t test; **, $P<0.01$, ****, $P<0.001$

The overall percentage of $CD4^+$ and $CD8^+$ T cells in the spleen and LN were comparable to the previous mixed chimeras (Figures 3.3A, 3.4A) and the bias of the $CD44^{-/-}$ progenitors towards developing into $CD4^+$ T cells remained (Figures 3.50B, 3.51B). When the CD4:CD8 ratio in the spleen was calculated there were approximately 4 $CD4^+$ T cells per $CD8^+$ T cell in the $CD44^{-/-}$ T cell population compared to 2 $CD4^+$ T cells per $CD8^+$ T cell in the donor ($Ly5.2^+$) $CD44^{+/+}$ population (Figures 3.50C, 3.4C). The CD4:CD8 ratio in the LN showed that there were approximately 2 $CD4^+$ T cells per $CD8^+$ T cell in the $CD44^{-/-}$ T cell population compared to 1.3 $CD4^+$ T cells per $CD8^+$ T cell in the donor ($Ly5.2^+$) $CD44^{+/+}$ population (Figure 3.51C). The bias previously found in the $CD4^+CD25^+$ T cell population in the mixed chimeras (Figure 3.5, 3.6), was again demonstrated in these 1100 chimeras (Figures 3.52, 3.53). There was again a significantly higher contribution from the $CD44^{-/-}$ cells in the $CD3^+$ $CD4^+$ $CD25^+$ subset than in the $CD3^+$ $CD4^+$ $CD25^-$ subset (Figure 3.52B, 3.53B). This was most dramatic in the LN of these mice where 30% of the $CD25^+CD4^+$ T cells were derived from the $CD44^{-/-}$ progenitors compared to 10% of the $CD25^-CD4^+$ T cells. There was also a difference in these chimeras by their having an overall lower $CD25^-CD4^+$: $CD25^+CD4^+$ in the spleens compared to the pervious mixed chimeras, previously the overall ratio of $CD25^-CD4^+$: $CD25^+CD4^+$ T cells in the spleens was 8, but in these mixed chimeras it was down to 4 $CD25^-CD4^+$ T cells to every $CD25^+CD4^+$ T cell.

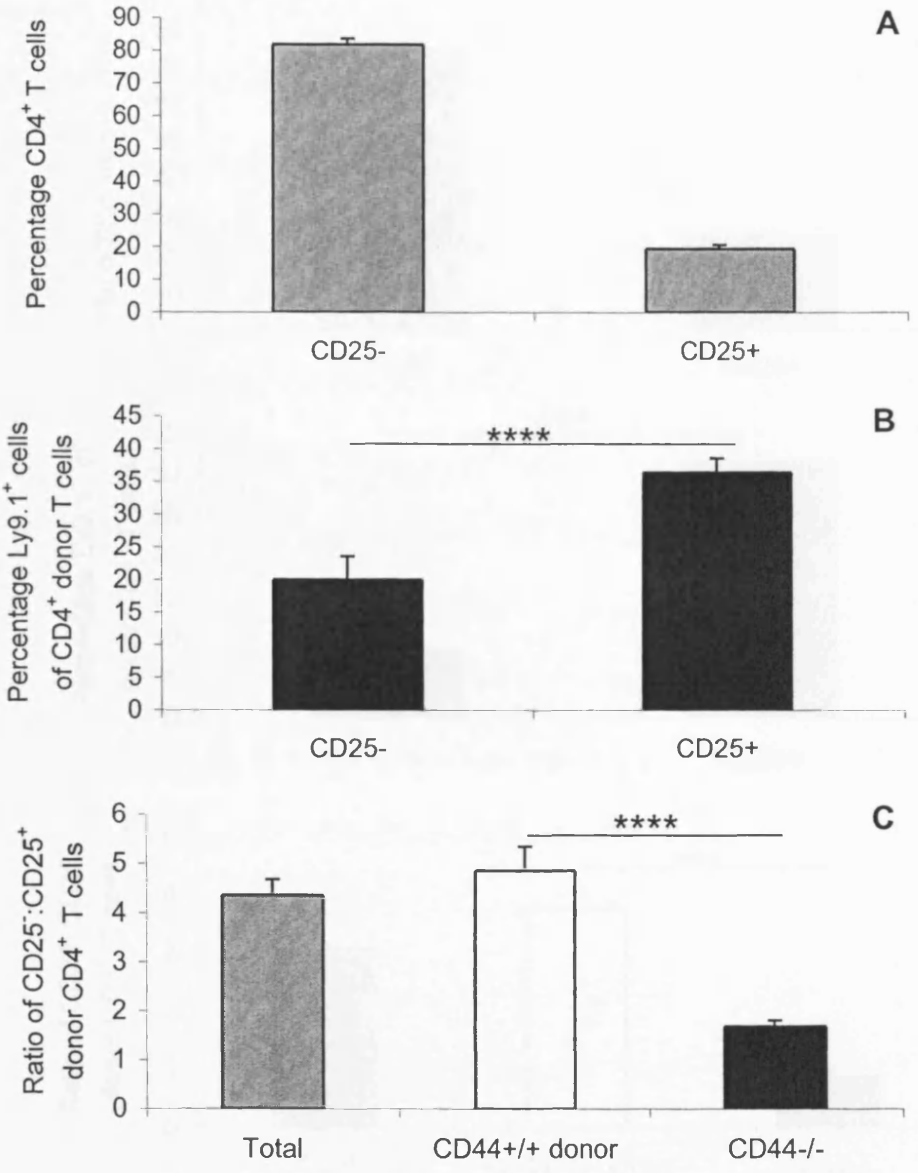

Figure 3.52. Analysis of CD4 T cell subsets in spleens of $CD44^{+/+}$ & $CD44^{-/-}\rightarrow CD44^{+/+}$ (1100 rads) chimeras.

A. Percentages of $CD25^{+}$ and $CD25^{-}$ $CD4^{+}$ T cells in spleens. B. The percentage of $Ly9.1^{+}$ cells in each donor $CD4^{+}$ T cell subset. C. The B:T cell ratio among total and $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 groups of 3. P values, 2-sample t test; ****, $P<0.001$

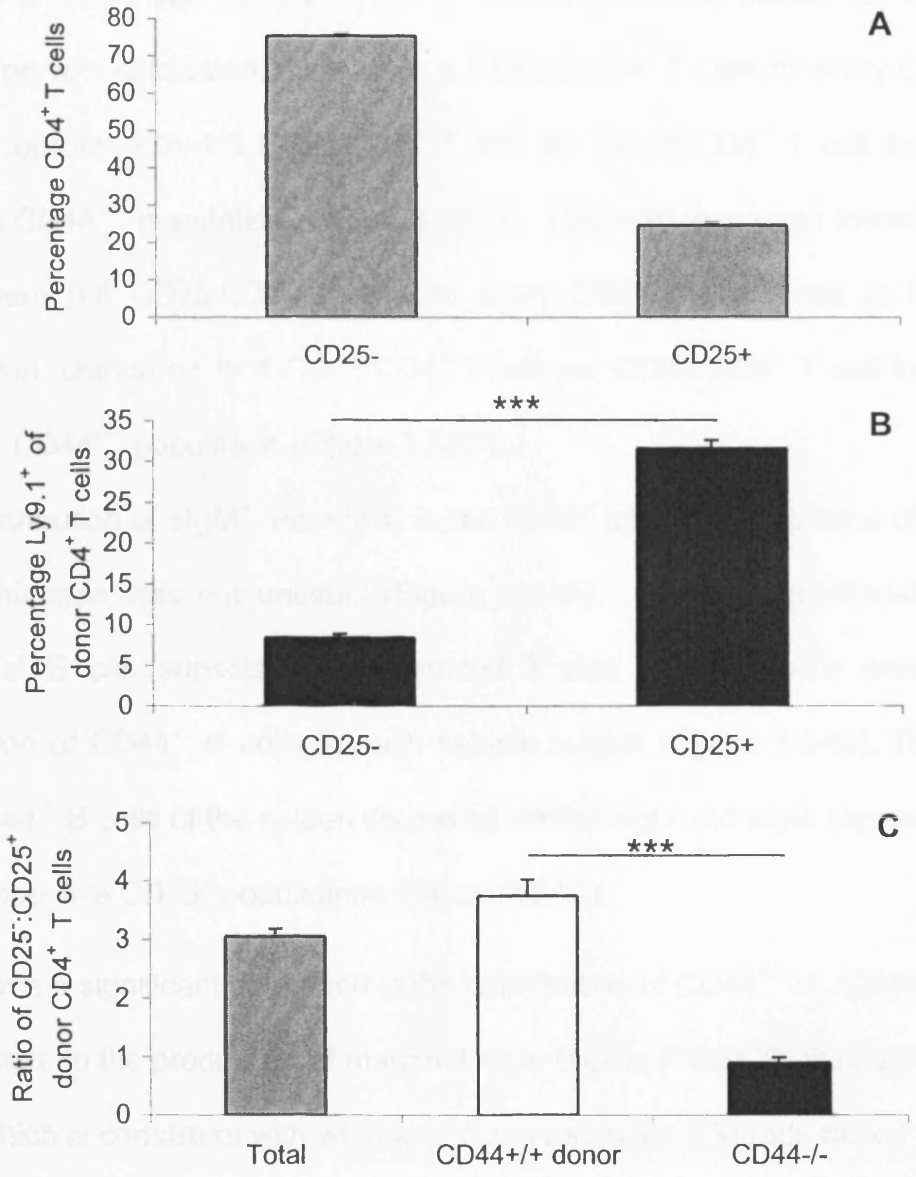

Figure 3.53. Analysis of $CD4^+$ T cell subsets in lymph nodes of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ (1100 rads) chimeras.

A. Percentages of $CD25^+$ and $CD25^-$ $CD4^+$ T cells in lymph nodes. B. The percentage of $Ly9.1^+$ cells in each donor $CD4^+$ T cell population. C. The B:T cell ratio among total and $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean ± s.e.m. obtained from 1 groups of 3 chimeras. P values, 2-sample t test; ***, $P<0.005$,

When the $CD25^-CD4^+$:$CD25^+$ $CD4^+$ T cell ratio in the spleen of the $CD44^{-/-}$ population was calculated, there were 1.5 $CD25^-CD4^+$ T cells to every $CD25^+CD4^+$ T cell, compared to 4.5 $CD25^-CD4^+$ T cell per $CD25^+CD4^+$ T cell in the donor ($Ly5.2^+$) $CD44^{+/+}$ population (Figure 3.52C). This ratio was even lower in the LN, there were 0.8 $CD25^-CD4^+$ T cells to every $CD25^+CD4^+$ T cell in the $CD44^{-/-}$ population, compared to 4 $CD25^-CD4^+$ T cell per $CD25^+CD4^+$ T cell in the donor ($Ly5.2^+$) $CD44^{+/+}$ population (Figure 3.53C).

The distribution of $sIgM^+$ and $sIgD^+$ in the $B220^+$ splenic populations of the mixed 1100 chimeras was not unusual (Figure 3.54A). When the composition of the individual B cell subsets was examined it was evident there was a similar proportion of $CD44^{-/-}$ B cells in each splenic subset (Figure 3.54B). The $CD44^{+/+}$ and $CD44^{-/-}$ B cells of the spleen displayed similar sIgD and sIgM expression within their respective $CD19^+$ populations (Figure 3.54C).

There was a significant difference in the contribution of $CD44^{-/-}$ vs. $CD44^{+/+}$ progenitors to the production of marginal zone B cells ($CD21^+CD23^-B220^+$) (Figure 3.55) which is consistent with what was observed in the 900 rads mixed chimeras. 36% of the marginal zone B cells were $CD44^{-/-}$ derived compared to 20% of the follicular B cells ($CD21^+CD23^+B220^+$) (Figure 3.55BA). This difference was also reflected in the distribution of marginal zone and follicular B cells within the $CD44^{+/+}$ and $CD44^{-/-}$ B cell populations. 7.7% of the $CD44^{+/+}$ B cell population were marginal zone B cells compared to 16% of the $CD44^{-/-}$ B cells (Figures 3.55C).

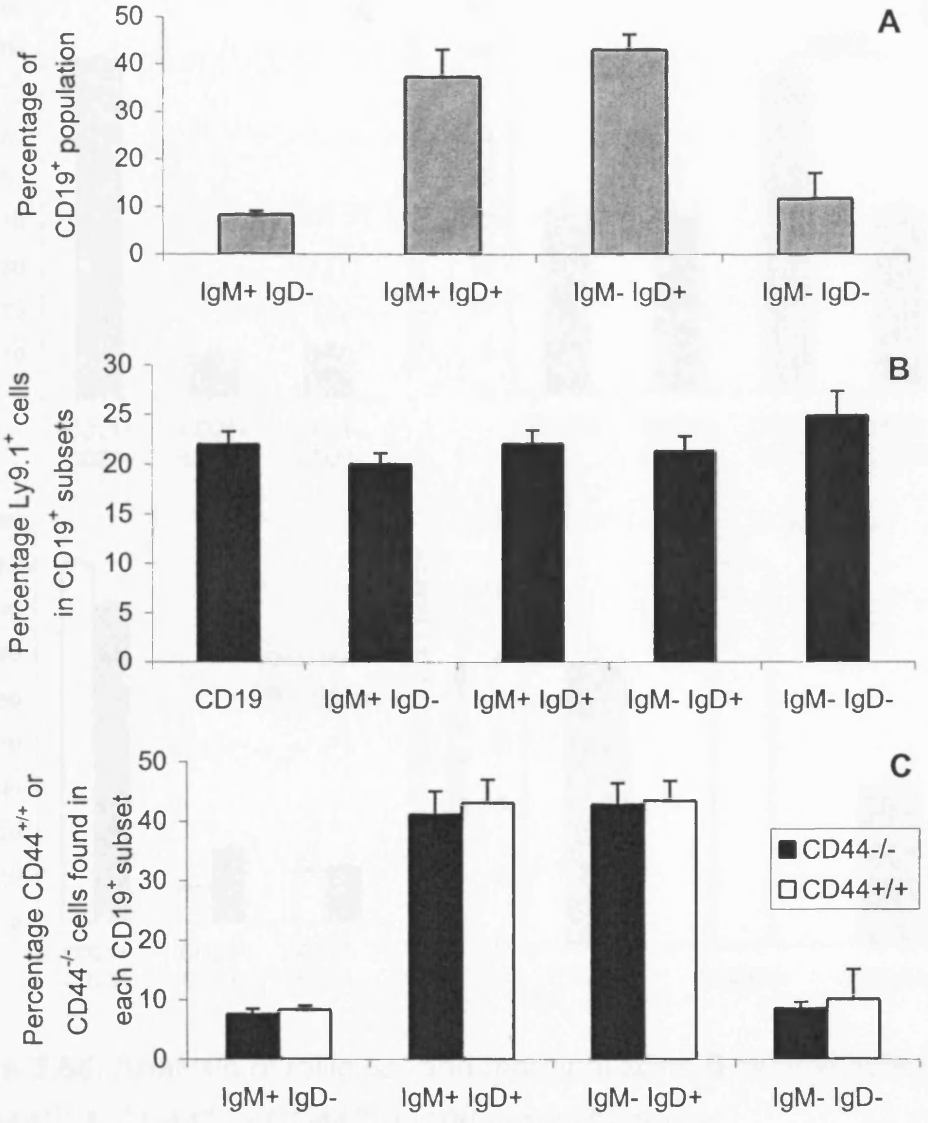

Figure 3.54. Analysis of B cell subsets in spleens of $CD44^{+/+}$ & $CD44^{-/-}$ → $CD44^{+/+}$ (1100 rads) chimeras.

A. Percentages of splenic B cell ($CD19^{+}$) subsets defined by sIgM and sIgD expression. B. The percentage of $Ly9.1^{+}$ cells in each B cell subset. Distribution of sIgM and sIgD staining among $CD44^{-/-}$ or $CD44^{+/+}$ $CD19^{+}$ cells. Data are mean ± s.e.m. obtained from 1 group of 2 and 2 groups of 3 mice.

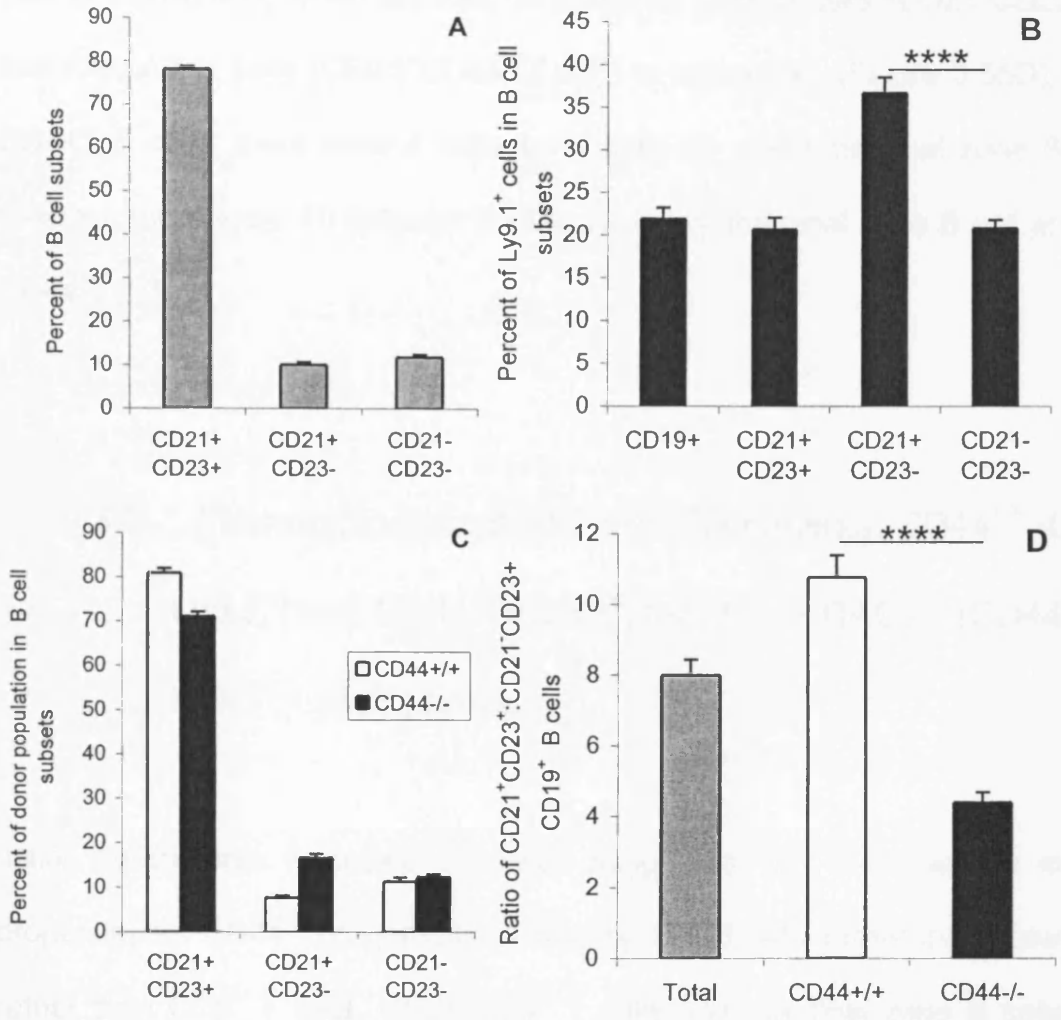

Figure 3.55. Analysis of follicular and marginal zone B cells in spleen of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ (1100 rads) chimeras.

A. Percentages of splenic B cell ($CD19^+$) subsets defined by CD21 and CD23. B. The percentage of $Ly9.1^+$ ($CD44^{-/-}$) donor cells in each B cell subset. D. Ratio of $CD21^+$ $CD23^+$: $CD21^+$ $CD23^-$ cells among total, $CD44^{-/-}$ and $CD44^{+/+}$ $CD19^+$ cells. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 groups of 3. P values, 2-sample t test; ****, $P<0.001$

This is highlighted, when the ratio of marginal zone B cells ($CD21^+CD23^-CD19^+$) and follicular B cells ($CD21^+CD23^+CD19^+$) is calculated, (Figure 3.55D). Among $CD44^{-/-}$ B cells, there were 4 follicular B cells for every marginal zone B cell. In contrast, there were 10 follicular B cells for every marginal zone B cell among the $CD44^{+/+}$ cells.

3.3.7. Phenotypic description of RAG chimeras: $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) and $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) hosts

Initial experiments in mixed chimeras suggested that there was a significant propensity for $CD44^{-/-}$ progenitors to develop into B cells rather than T cells, $CD4^+$ rather than $CD8^+$ T cells, $CD25^+CD4^+$ T cells and marginal zone B cells. Since some of this bias could have been exaggerated by the presence of contaminating host T cells, chimeras were made with a higher dose of irradiation 1100 rads, as opposed to 900 rads to try and eliminate the majority of the host T cells. In the resultant high dose chimeras, the $CD44^{-/-}$ progenitors still showed a biased tendency to produce B cells, $CD4^+$ T cells, $CD25^+CD4^+$ T cells and marginal zone B cells. However, although host cells were distinguished from donor cells in the analysis, there were still radioresistant T cells present in the 1100 rads chimeras. In addition, the use of higher doses of irradiation could cause damage to the stromal cell layer in the bone marrow and thymus which is necessary for the development of B and T cells. Therefore to eliminate all host T cells and the

possibility that they could interfere with the seeding and development of the donor bone marrow, it was decided to create the chimeras in $RAG^{-/-}$ hosts. $RAG^{-/-}$ mice lack the enzyme RAG2 which is responsible for initiating double stranded DNA breaks, and therefore all T and B cell development is aborted. Hence, these chimeras will only have mature T and B cells, which have been derived from cells in the donor bone marrow.

$Ly5.2^{+/+}$ and $CD44^{-/-}$ mice were sacrificed and their bone marrow T cell depleted by antibody mediated complement cytotoxicity. The cells were mixed in a 1:1 ratio and injected into irradiated host $RAG^{-/-}$ mice (400 rads). The host mice were given a lower dose of irradiation, for there are no mature lymphocytes to eliminate, and all is required is to provide 'space' in the bone marrow for the donor bone marrow to fill. The lower dose of irradiation causes less damage to the stromal cells. Once immune reconstitution had occurred the mice were sacrificed, and their phenotype was analysed by FACS. Cells derived from the $CD44^{-/-}$ progenitor cells were identified using an anti-Ly5.1 (CD45.2) antibody.

These mice had normal cell numbers in spleens and LN (appendix 1), and overall similar percentages of T and B cells (Figures 3.56A, 3.57A). When the T and B cells from the $CD44^{-/-}$ host chimeras were analysed there was a significant difference in the origin of the T and B cells (Figure 3.56B, 3.57B).

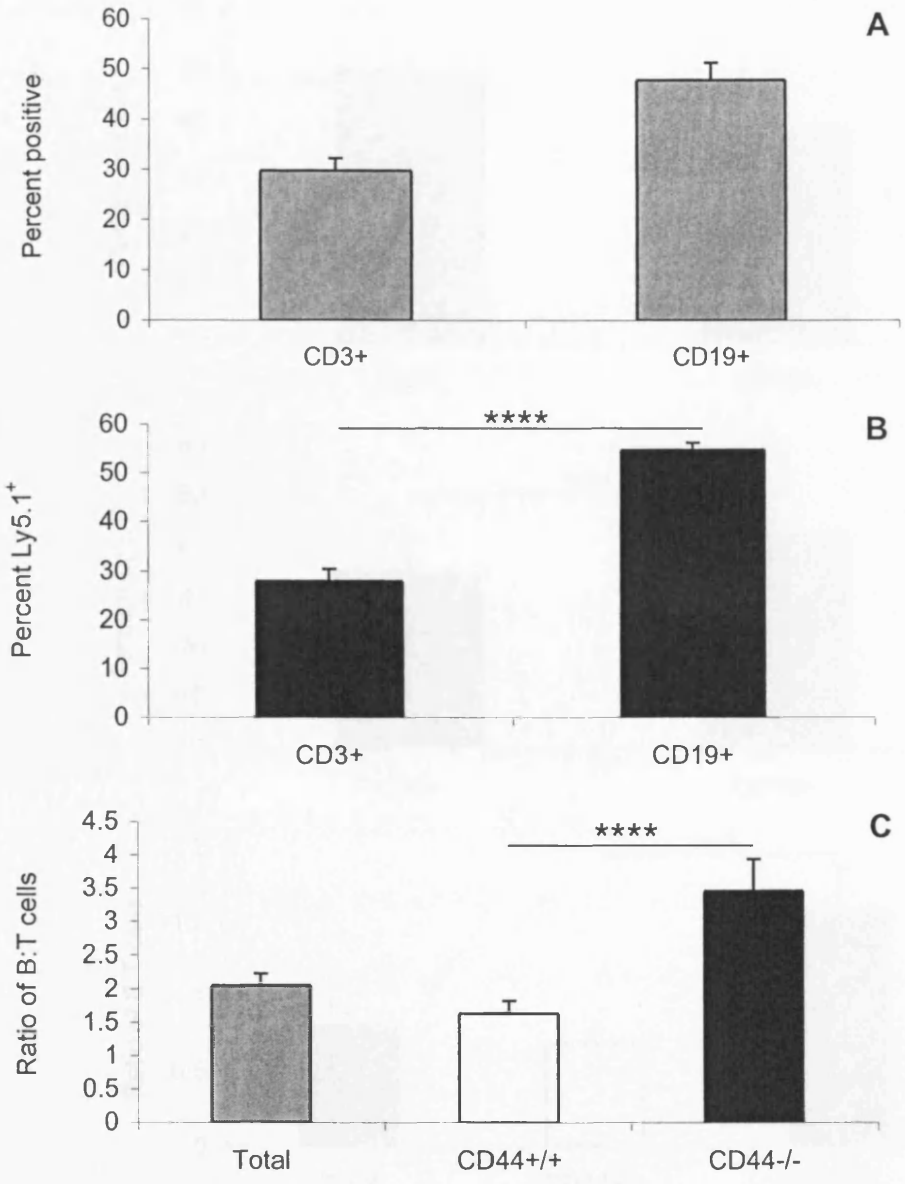

Figure 3.56. Analysis of T and B cells in spleens of $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

A. Percentages of T and B cells in spleen. B. The percentage of $Ly5.1^{+}$ cells in each splenic population. C. The B:T cell ratio among total, $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 groups of 3. P values, 2-sample t test; ****, $P<0.005$

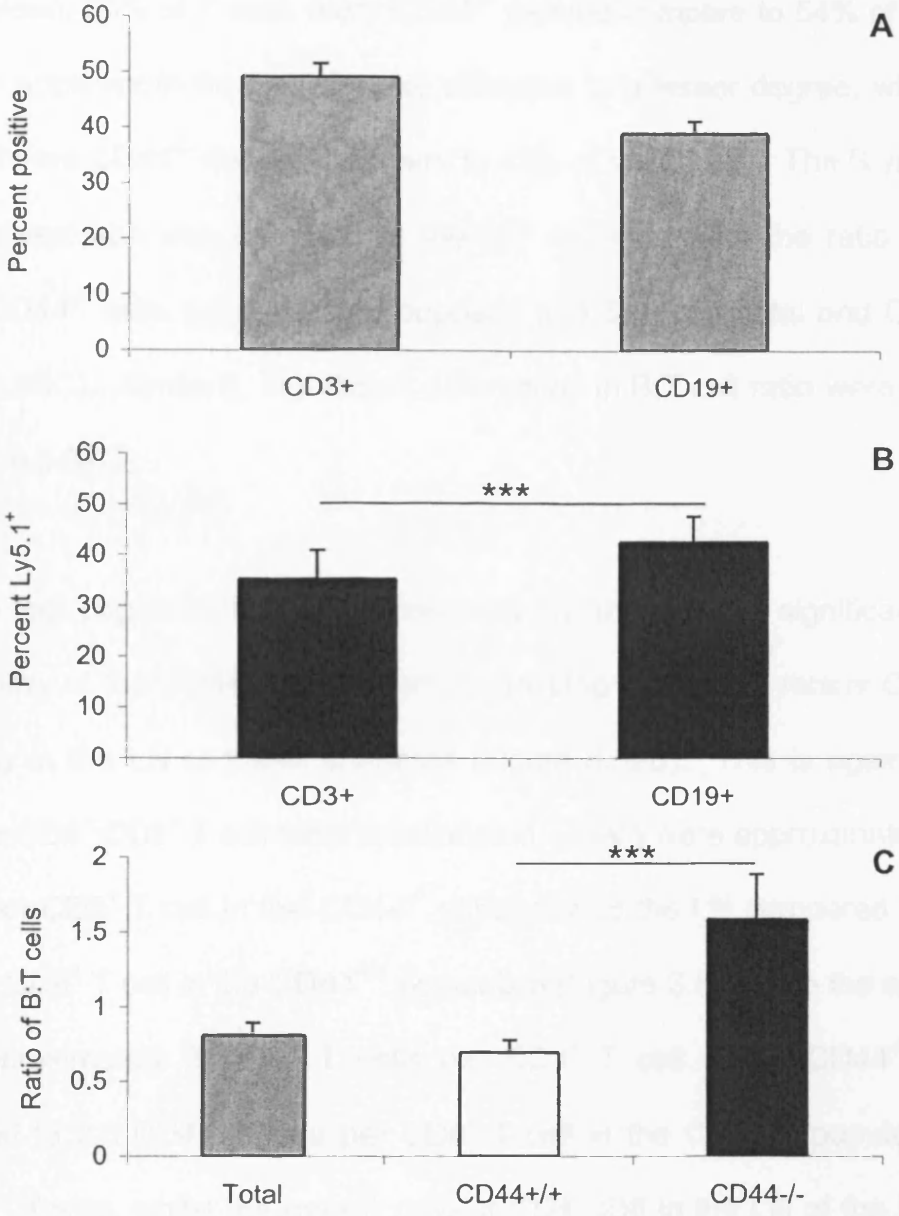

Figure 3.57. Analysis of T and B cells in lymph nodes of $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

A. Percentages of T and B cells in lymph nodes. B. The percentage of $Ly9.1^{+}$ cells in each population. C. The B:T cell ratio among total, $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 groups of 3. P values, 2-sample t test; ***, $P<0.005$,

In the spleen, 28% of T cells were $CD44^{-/-}$ derived compare to 54% of the B. This was also apparent in the LN of these chimeras to a lesser degree, where 29% of the T cells are $CD44^{-/-}$ derived compare to 42% of the B cells. The B vs. T cell bias in the spleen was also reflected in the B:T cell ratio, with the ratio of B:T cells among $CD44^{-/-}$ cells being 3.2, as opposed to 1.5 among total and $CD44^{+/+}$ cells (Figure 3.56C). Similarly, significant differences in B:T cell ratio were observed in LN (Figure 3.57C).

Among T cell populations in the spleen and LN, there was a significant difference in the ability of the $CD44^{-/-}$ progenitors to develop into $CD4^+$ versus $CD8^+$ T cells, especially in the LN of these chimeras (Figure 3.59B). This is again highlighted when the $CD4^+$:$CD8^+$ T cell ratio is calculated. There were approximately 1.5 $CD4^+$ T cells per $CD8^+$ T cell in the $CD44^{-/-}$ population of the LN compared to 1 $CD4^+$ T cells per $CD8^+$ T cell in the $CD44^{+/+}$ population (Figure 3.59C). In the spleens there were approximately 3 $CD4^+$ T cells per $CD8^+$ T cell in the $CD44^{-/-}$ population compared to 2.5 $CD4^+$ T cells per $CD8^+$ T cell in the $CD44^{+/+}$ population (Figure 3.58C). Of note, whilst the overall ratio of CD4:CD8 in the LN of the $RAG^{-/-}$ hosts was 1, in the $CD44^{+/+}$ hosts it has been consistently approximately 1.5, the splenic CD4: CD8 ratio in the $RAG^{-/-}$ hosts is consistent with that in other mixed chimeras. The bias in the $CD25^+CD4^+$ T cell population found in the $CD44^{+/+}$ host mice (Figures 3.52& 3.53, 3.5 & 3.6), was also observed in the $RAG^{-/-}$ host mixed chimeras (Figure 3.60, 3.61).

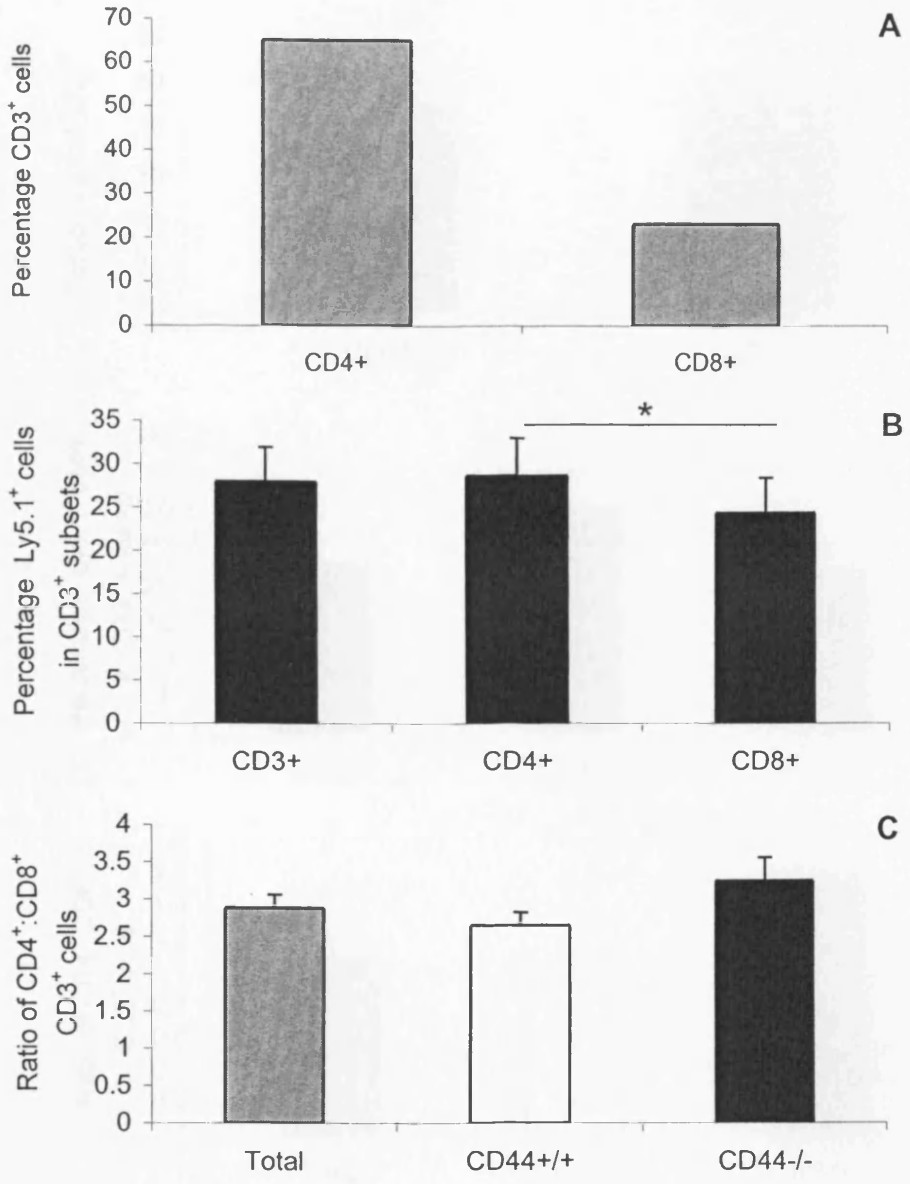

Figure 3.58. Analysis of T cell subsets in spleens of $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) $\rightarrow$ $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

A. Percentages of $CD4^{+}$ and $CD8^{+}$ T cells in spleens. B. The percentage of $Ly9.1^{+}$ cells in each donor T cell population. C. The B:T cell ratio among total, $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean $\pm$ s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 group of 3. P values, 2-sample t test; *, $P<0.05$

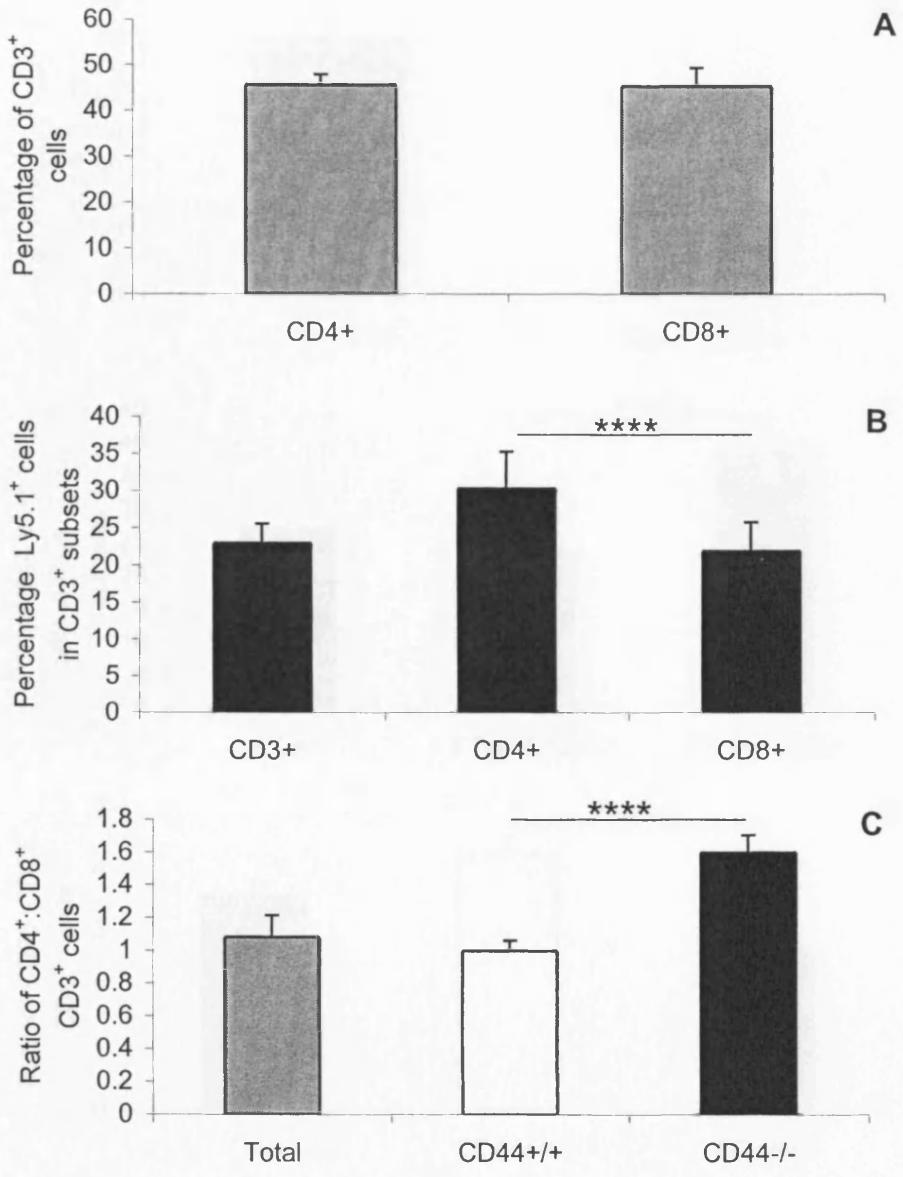

Figure 3.59. Analysis of T cell subsets in lymph nodes of $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

A. Percentages of $CD4^{+}$ and $CD8^{+}$ T cells in lymph nodes. B. The percentage of $Ly9.1^{+}$ cells in each donor T cell population. C. The B:T cell ratio among total, $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 groups of 3. P values, 2-sample t test; ****, $P<0.001$

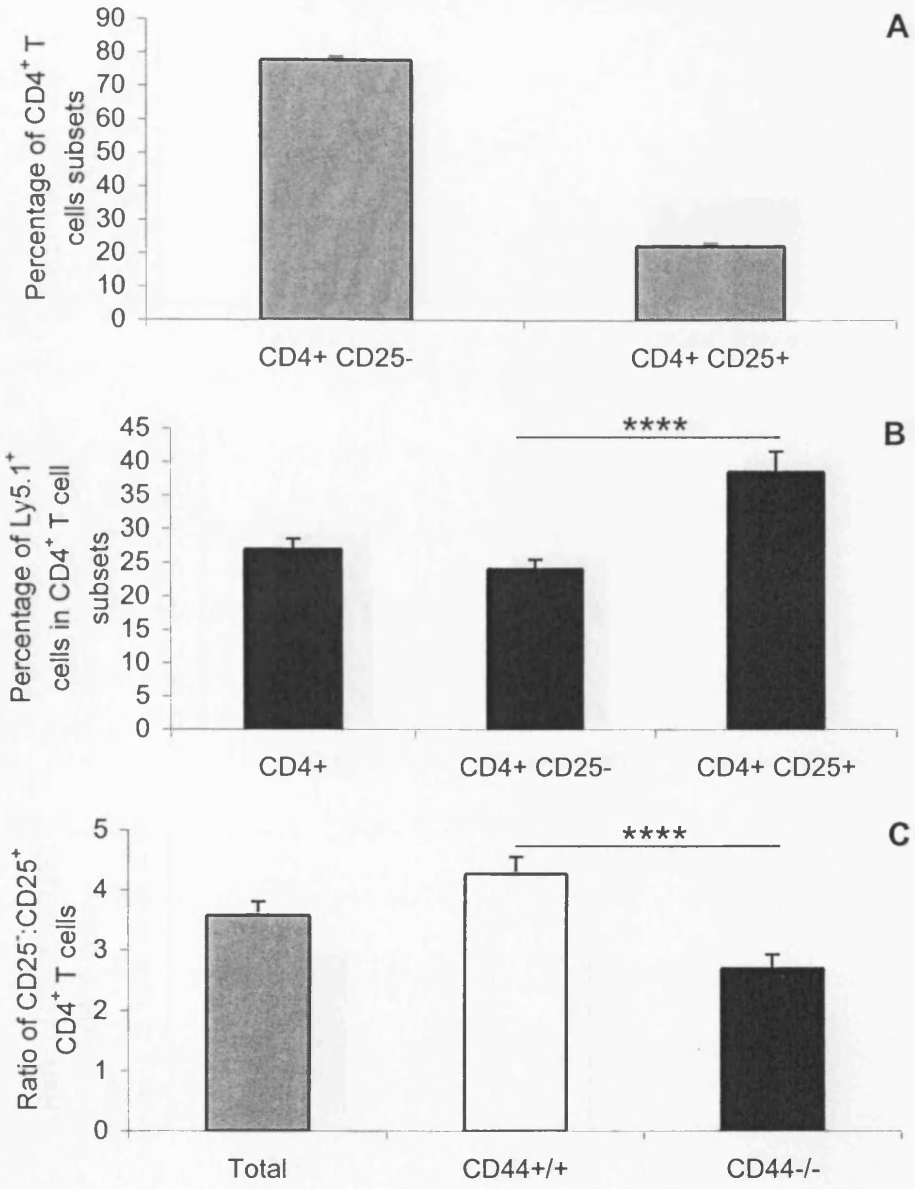

Figure 3.60. Analysis of CD4 T cell subsets in spleens of $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

A. Percentages of $CD25^{+}$ and $CD25^{-}$ $CD4^{+}$ T cells in spleens. B. The percentage of $Ly9.1^{+}$ cells in each donor $CD4^{+}$ T cell subset. C. The B:T cell ratio among total, $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 groups of 3. P values, 2-sample t test; ****, $P<0.001$

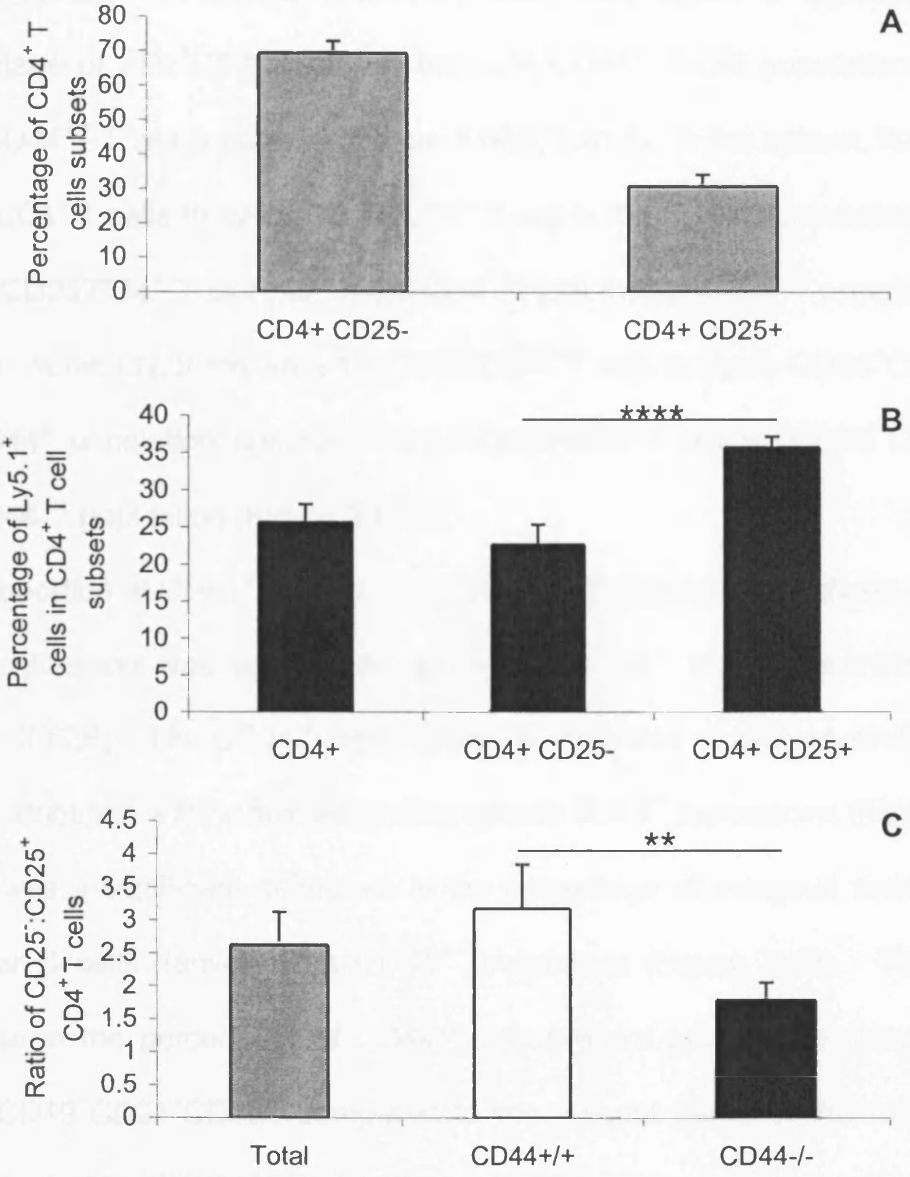

Figure 3.61. Analysis of $CD4^+$ T cell subsets in lymph nodes of $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras.

A. Percentages of $CD25^+$ $CD25^-$ $CD4^+$ T cells in lymph nodes. B. The percentage of $Ly9.1^+$ cells in each donor $CD4^+$ T cell population. C. The B:T cell ratio among total, $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 groups of 3. P values, 2-sample t test; **, $P<0.01$, ****, $P<0.001$

In the $RAG^{-/-}$ host mixed chimeras, there was again a significantly higher percentage of $CD3^{+}CD4^{+}CD25^{+}$ in the $CD4^{+}CD44^{-/-}$ T cell population than in the $CD4^{+}CD44^{+/+}$ T cell population (Figure 3.60B, 3.61B). In the spleen, there were 2.7 $CD25^{-}CD4^{+}$ T cells to every $CD25^{+}CD4^{+}$ T cell in the $CD44^{-/-}$ population, compared to 3.9 $CD25^{-}CD4^{+}$ T cell per $CD25^{+}CD4^{+}$ T cell in the $CD44^{+/+}$ population (Figure 3.60C). In the LN, there were 1.6 $CD25^{-}CD4^{+}$ T cells to every $CD25^{+}CD4^{+}$ T cell in the $CD44^{-/-}$ population, compared to 2.6 $CD25^{-}CD4^{+}$ T cell per $CD25^{+}CD4^{+}$ T cell in the $CD44^{+/+}$ population (Figure 3.61C).

The proportion of $CD44^{-/-}$ B cells within B cell sub populations defined by sIgM and sIgD expression was again constant in the $RAG2^{-/-}$ host mice mixed chimeras (Figure 3.62B). The $CD44^{+/+}$ and $CD44^{-/-}$ B cells also displayed similar sIgD and sIgM distribution within their respective splenic $CD19^{+}$ populations (Figure 3.62C).

There was a significant difference in the percentage of marginal zone B cells vs. follicular B cells derived from $CD44^{-/-}$ progenitors (Figure 3.63). There was an increase in the percentage of $CD44^{-/-}$ cells present among the marginal zone B cells ($CD19^{+}CD21^{+}CD23^{-}$) compared to the amount found in the follicular B cell population ($CD19^{+}CD21^{+}CD23^{+}$) (Figure 3.63B). This observation is reinforced by a higher marginal zone frequency in the $CD44^{-/-}$ B cell population. Thus there were 2.4 $CD44^{-/-}$ follicular B cell to every $CD44^{-/-}$ marginal zone B cell, compared to 5 $CD44^{+/+}$ follicular B cell to every $CD44^{+/+}$ marginal zone B cell (Figure 3.63C).

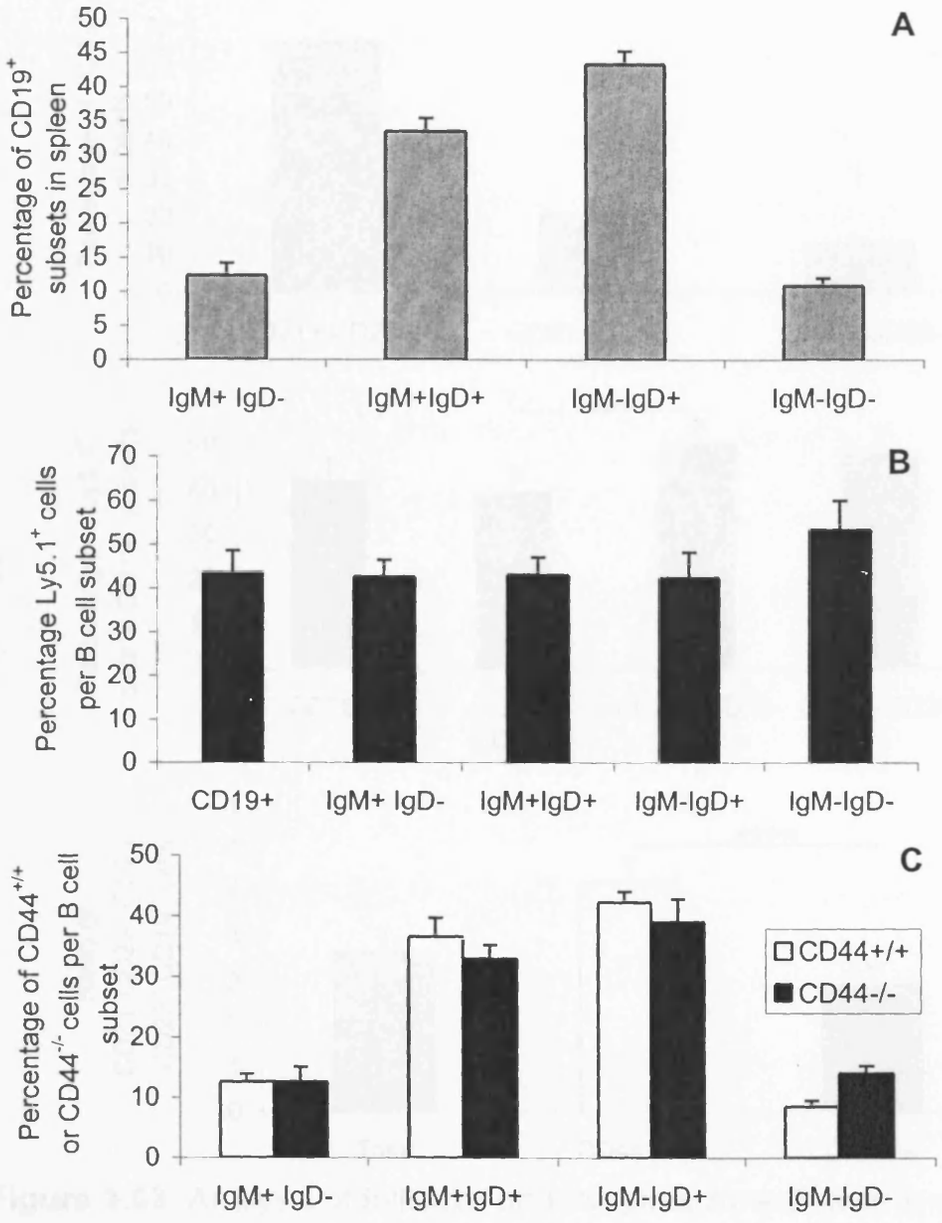

Figure 3.62. Analysis of B cell subsets in spleens of $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) $\rightarrow$ $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

A. Percentages of splenic B cell ($CD19^{+}$) subsets defined by sIgM and sIgD expression. B. The percentage of $Ly9.1^{+}$ cells in each B cell subset. Distribution of sIgM and sIgD staining among $CD44^{-/-}$ or $CD44^{+/+}$ $CD19^{+}$ cells. Data are mean $\pm$ s.e.m. obtained from 1 group of 2 and 2 groups of 3 mice.

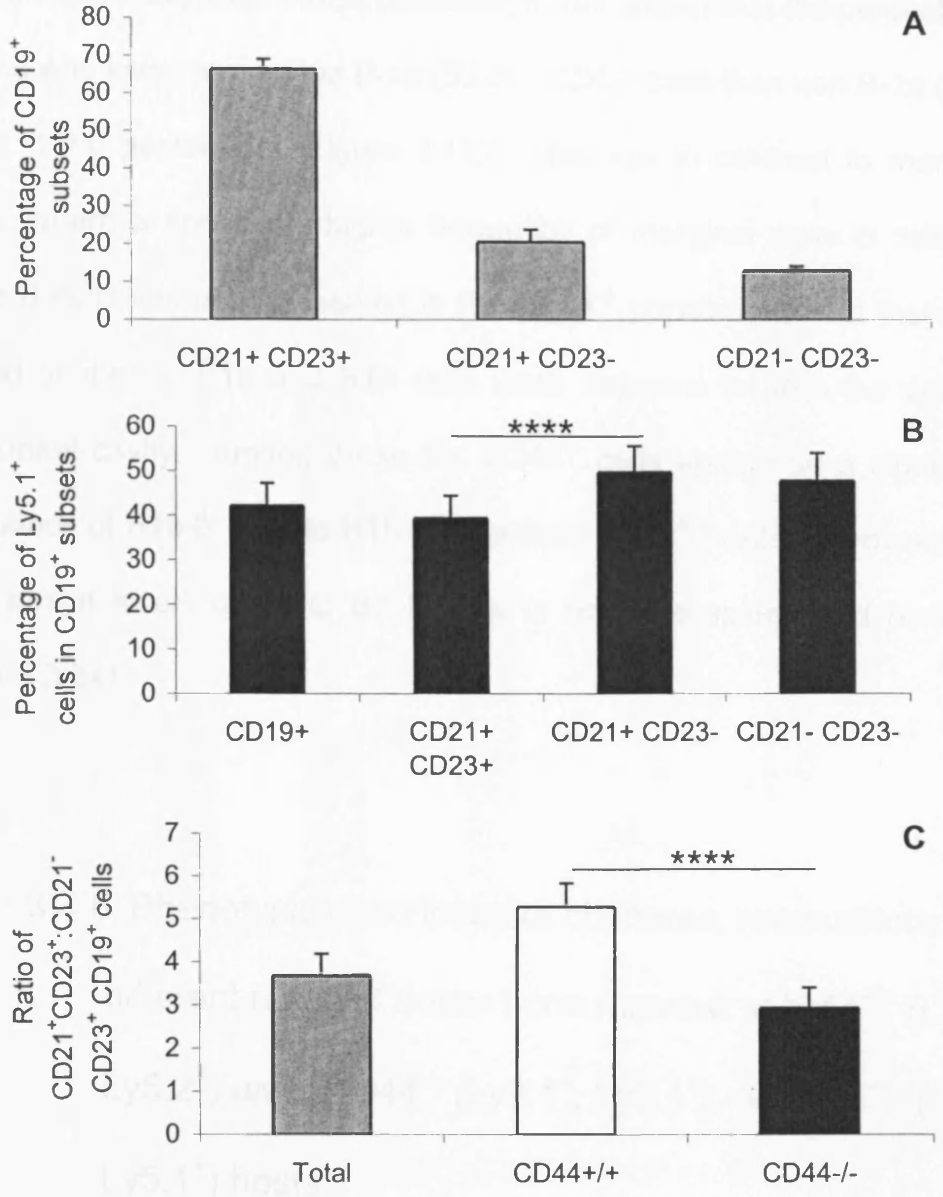

Figure 3.63. Analysis of follicular and marginal zone B cells in spleens of $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

A. Percentages of splenic B cell ($CD19^{+}$) subsets defined by CD21 and CD23 expression. B. The percentage of $Ly9.1^{+}$ ($CD44^{-/-}$) donor cells in each B cell subset. C. Ratio of $CD21^{+}$ $CD23^{+}$: $CD21^{+}$ $CD23^{-}$ cells among total, $CD44^{-/-}$ and $CD44^{+/+}$ $CD19^{+}$ cells. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 groups of 3. P values, 2-sample t test; ****, $P<0.001$.

In our initial analysis of mixed chimeras, it was shown that the percentage of $CD44^{-/-}$ cells was lower among the B-1a ($B220^{+}$, $CD5^{+}$) cells than non B-1a ($B220^{+}$, $CD5^{-}$) cells in the peritoneum (Figure 3.10). This was in contrast to marginal zone B cells, where a constantly higher frequency of marginal zone B cells to follicular zone B cells has been observed in the $CD44^{-/-}$ populations. In these $RAG^{-/-}$ host mixed chimeras, B1a and B1b cells were detected in both the spleen and the peritoneal cavity. Among these the $CD44^{-/-}$ cells again had a significantly lower frequency of B1a-B cells to B1b-B cells than $CD44^{+/+}$ cells, but overall had similar had similar levels of B1 to B2 B cells in both the spleen and peritoneal cavity (Figure 3.64).

3.3.8. Phenotypic description of chimeras, reconstituted with different ratios of donor bone marrow: $xCD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) and $yCD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.1^{+}$) hosts.

Previous experiments have shown a putative role for CD44 in B cell and T cell development, and especially in the development of marginal zone B cells and $CD4^{+}$ and $CD4^{+}$ $CD25^{+}$ T cells. Since we have shown that this biased lymphocyte development occurs in chimeras made with $RAG^{-/-}$ hosts, the phenomenon is not related to the presence of host T cells.

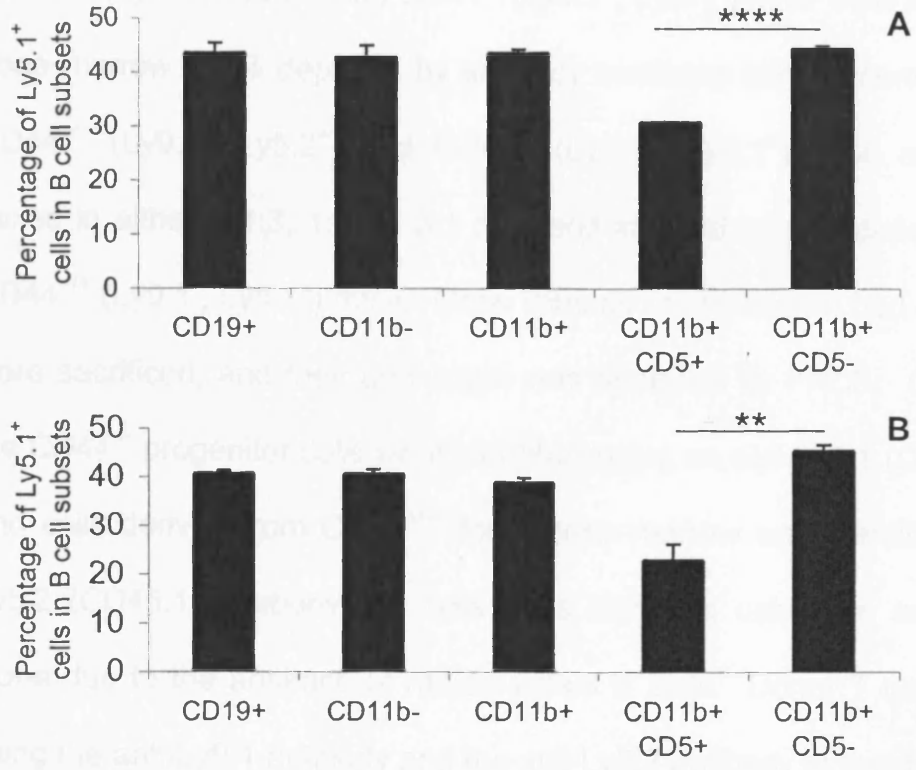

Figure 3.64. Analysis of B1 and B2 cells in spleen and peritoneal cavity of $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

A. Percentage of $Ly5.1^{+}$ cells in each B cell ($CD19^{+}$) subset of spleen.
B. Percentage of $Ly5.1^{+}$ cells in each B cell ($CD19^{+}$) subset of peritoneal cavity. Data are mean ± s.e.m. obtained from 1 group of 2 mice. P values, 2-sample t test; **, $P<0.01$, ****, $P<0.001$.

To determine whether biased development of $CD44^{-/-}$ lymphocytes depends on the extent of competition between $CD44^{-/-}$ and $CD44^{+/+}$ progenitors, we made a series of chimeras using different ratios of donor bone marrow.

$CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) and $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) mice were sacrificed and their bone marrow T cell depleted by antibody mediated complement cytotoxicity. The $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) and $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) bone marrow cells were mixed in either a 1:3, 1:1, or 3:1 ratio and injected into irradiated (900 rads) host $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.1^+$) mice. Once immune reconstitution had occurred the mice were sacrificed, and their phenotype was analysed by FACS. Cells derived from the $CD44^{-/-}$ progenitor cells were identified using an anti-Ly9.1 (CD229.1) antibody, and cells derived from $CD44^{+/+}$ donor bone marrow was identified using an anti-Ly5.2 (CD45.1) antibody. B cells were identified using the anti-Ly9.1 antibody alone due to the absence of radioresistant B cells. Donor T cells were identified using the anti-Ly9.1 antibody and the anti-Ly5.2 antibody or in some instances with an anti-$Ly5.1^+$ antibody and an anti-$Ly9.1^+$ antibody; in this case $Ly5.1^-$ lymphocytes were assumed to be $Ly5.2^+$ $CD44^{+/+}$ donor cells and $Ly5.1^+$ $Ly9.1^+$ cells were determined to be $Ly5.1^+$ $CD44^{-/-}$ donor cells.

These mice had normal cell numbers in their spleens and LN, and consistent percentages of total T and B cells, (Figures 3.65A, 3.66A).

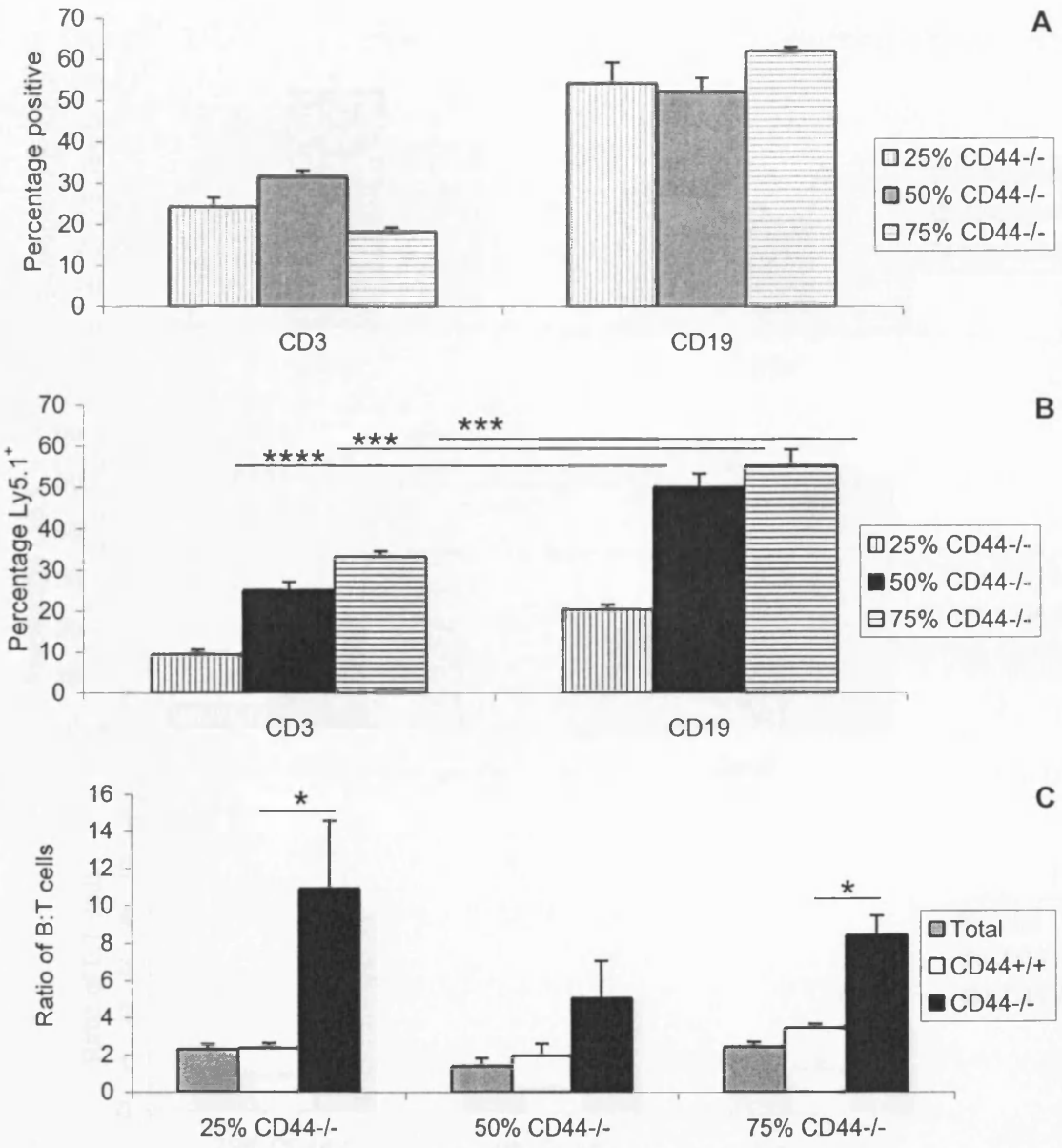

Figure 3.65. Analysis of T and B cells in the spleens of xCD44$^{+/+}$ & yCD44$^{-/-}$ → CD44$^{+/+}$ chimeras.

A. Percentages of T and B cells in the spleens in the various chimeras. B. The percentage of Ly9.1^{+} cells in each splenic population per chimeric group. C. The B:T cell ratio among CD44$^{-/-}$ and donor CD44$^{+/+}$ populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 8, 25% CD44$^{-/-}$, 1 group of 4, 50% CD44$^{-/-}$, 1 group of 6, 75% CD44$^{-/-}$. P values, 2-sample t test; *, P<0.05, ***, P<0.005, ****, P<0.001.

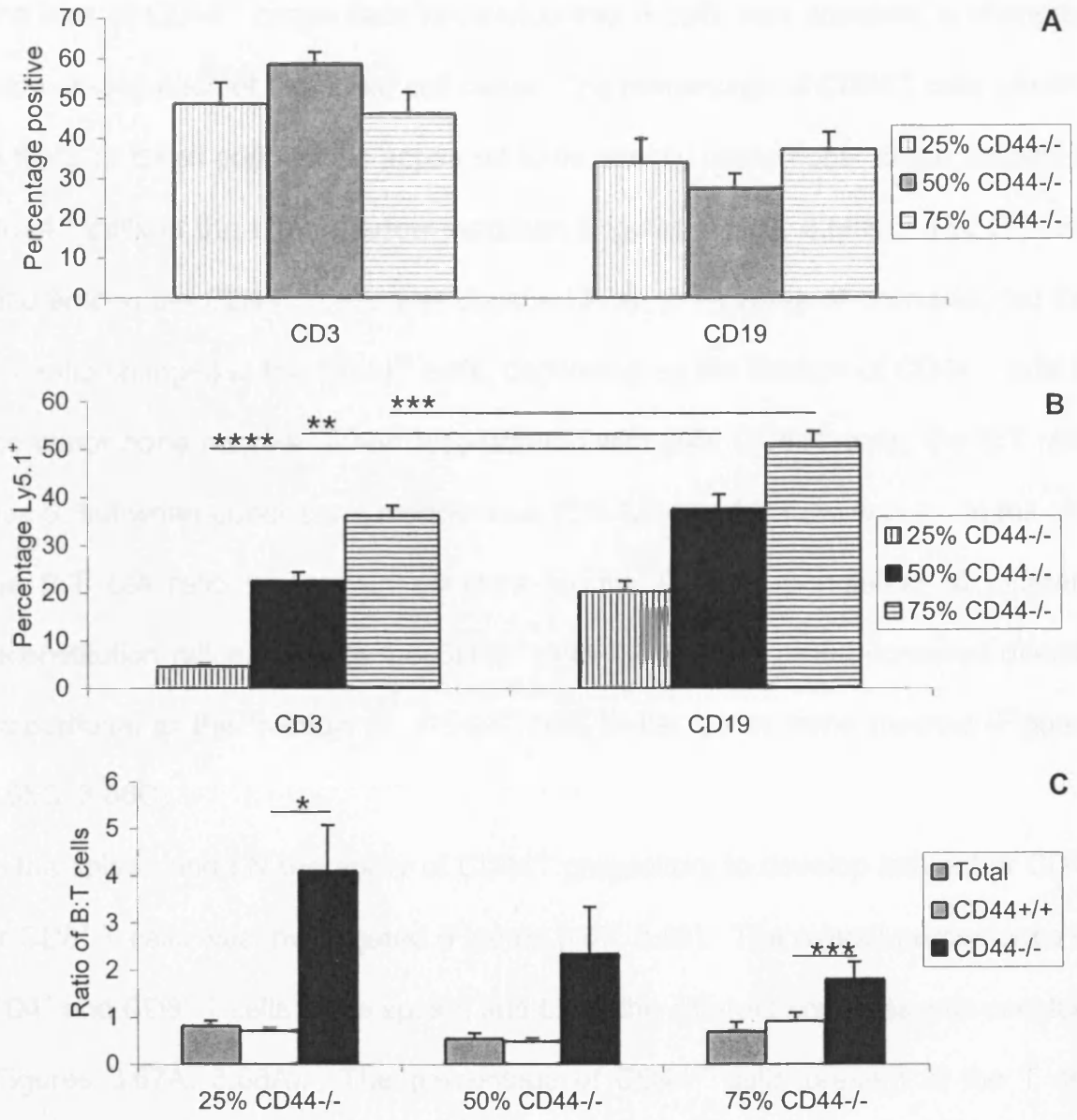

Figure 3.66. Analysis of T and B cells in the lymph nodes of xCD44$^{+/+}$ & yCD44$^{-/-}$→ CD44$^{+/+}$ chimeras.

A. Percentages of T and B cells in the lymph nodes in the various chimeras. B. The percentage of Ly9.1^{+} cells in each splenic population per chimeric group. C. The B:T cell ratio among CD44$^{-/-}$ and donor CD44$^{+/+}$ populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 8, 25% CD44$^{-/-}$, 1 group of 4, 50% CD44$^{-/-}$, 1 group of 6, 75% CD44$^{-/-}$. P values, 2-sample t test; *, P<0.05, **, P<0.01, ***, P<0.005, ****, P<0.001.

The bias of $CD44^{-/-}$ progenitors to develop into B cells was apparent in chimeras generated at each of the donor cell ratios. The percentage of $CD44^{-/-}$ cells present in the T or B cell populations appeared to be directly proportional to the fraction of $CD44^{-/-}$ cells in the bone marrow inoculum (Figures 3.65B, 3.66B). The B:T cell ratio among the $CD44^{+/+}$ cells was constant in all three types of chimeras, but the B:T ratio changed in the $CD44^{-/-}$ cells, depending on the fraction of $CD44^{-/-}$ cells in the donor bone marrow. When reconstituted with 25% $CD44^{-/-}$ cells, the B:T ratio was 5, but when donor bone marrow was 75% $CD44^{-/-}$ this ratio was 7. In the LN, the B:T cell ratio was again constant for the $CD44^{+/+}$ population at different reconstitution ratios, while in the $CD44^{-/-}$ cells the B:T cell ratio decreased directly proportional to the fraction of $CD44^{-/-}$ cells in the donor bone marrow (Figures 3.65C, 3.66C).

In the spleen and LN the ability of $CD44^{-/-}$ progenitors to develop into either $CD4^+$ or $CD8^+$ T cells was investigated (Figures 3.67, 3.68). The overall percentages of $CD4^+$ and $CD8^+$ T cells in the spleen and LN of the different chimeras was constant (Figures 3.67A, 3.68A). The percentage of $CD44^{-/-}$ cells present in the T cell subsets increased in direct proportion with the fraction of $CD44^{-/-}$ cells in the donor bone marrow. A higher CD4:CD8 ratio among $CD44^{-/-}$ vs. $CD44^{+/+}$ T cells was observed in all chimeras (Figures 3.67B, 3.68B), and this did not correlate with the ratio of $CD44^{-/-}$ cells in donor marrow (Figures 3.67C, 3.68C). The bias previously found in the $CD25^+CD4^+$ T cell population in the mixed chimeras (Figure 3.5 & 3.6, 3.52 & 5.53) was again demonstrated in spleens of these chimeras (Figure 3.69).

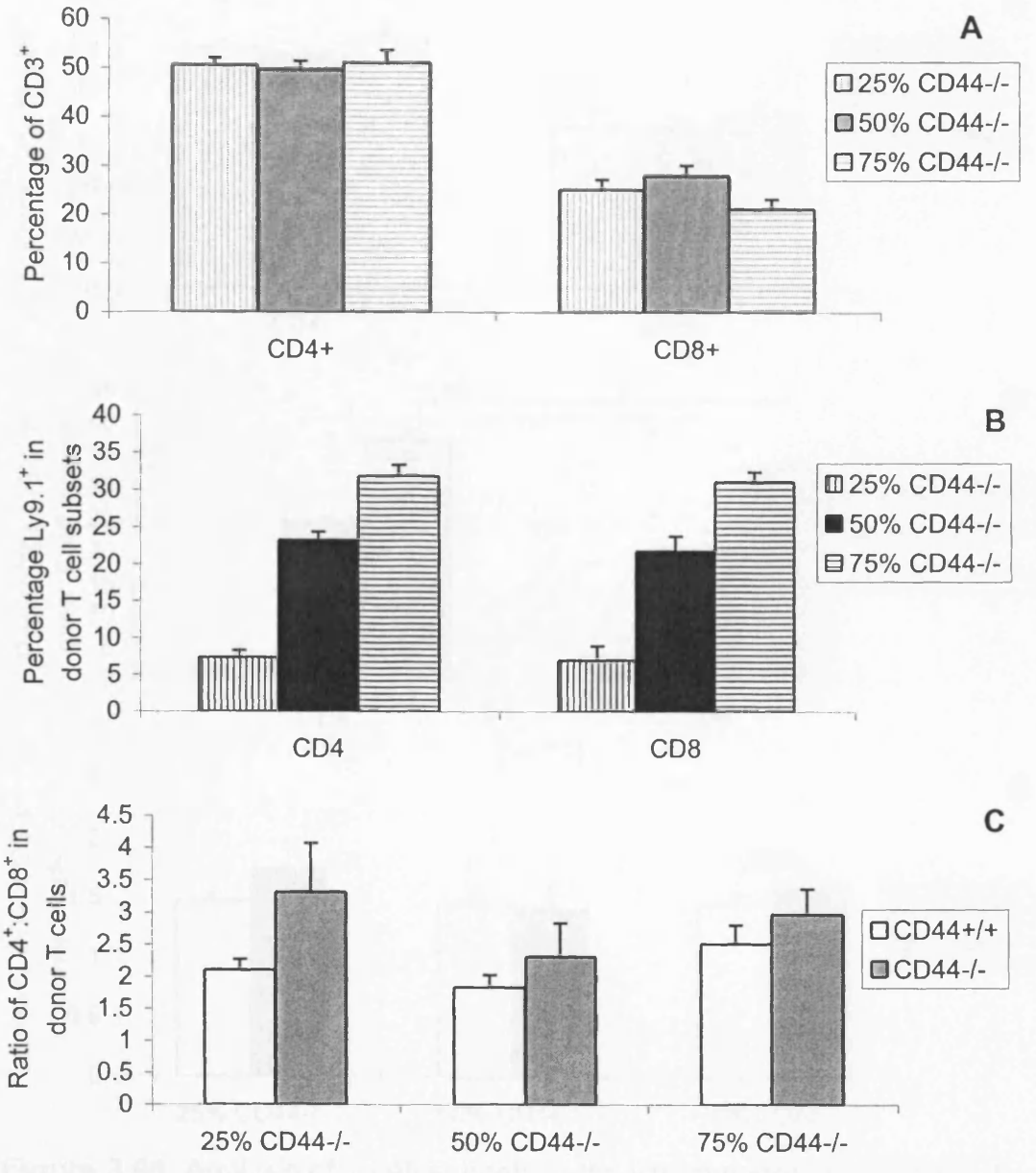

Figure 3.67. Analysis of T cell subsets in the spleens of xCD44$^{+/+}$ & yCD44$^{-/-}$→ CD44$^{+/+}$ chimeras.

A. Percentages of CD4^{+} and CD8^{+} T cells in the spleens of the various chimeras. B. The percentage of Ly9.1^{+} cells in each splenic donor T cell subset population per chimeric group. C. The CD4^{+}:CD8^{+} T cell ratio among CD44$^{-/-}$ and donor CD44$^{+/+}$ populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 8, 25% CD44$^{-/-}$, 1 group of 4, 50% CD44$^{-/-}$, 1 group of 6, 75% CD44$^{-/-}$.

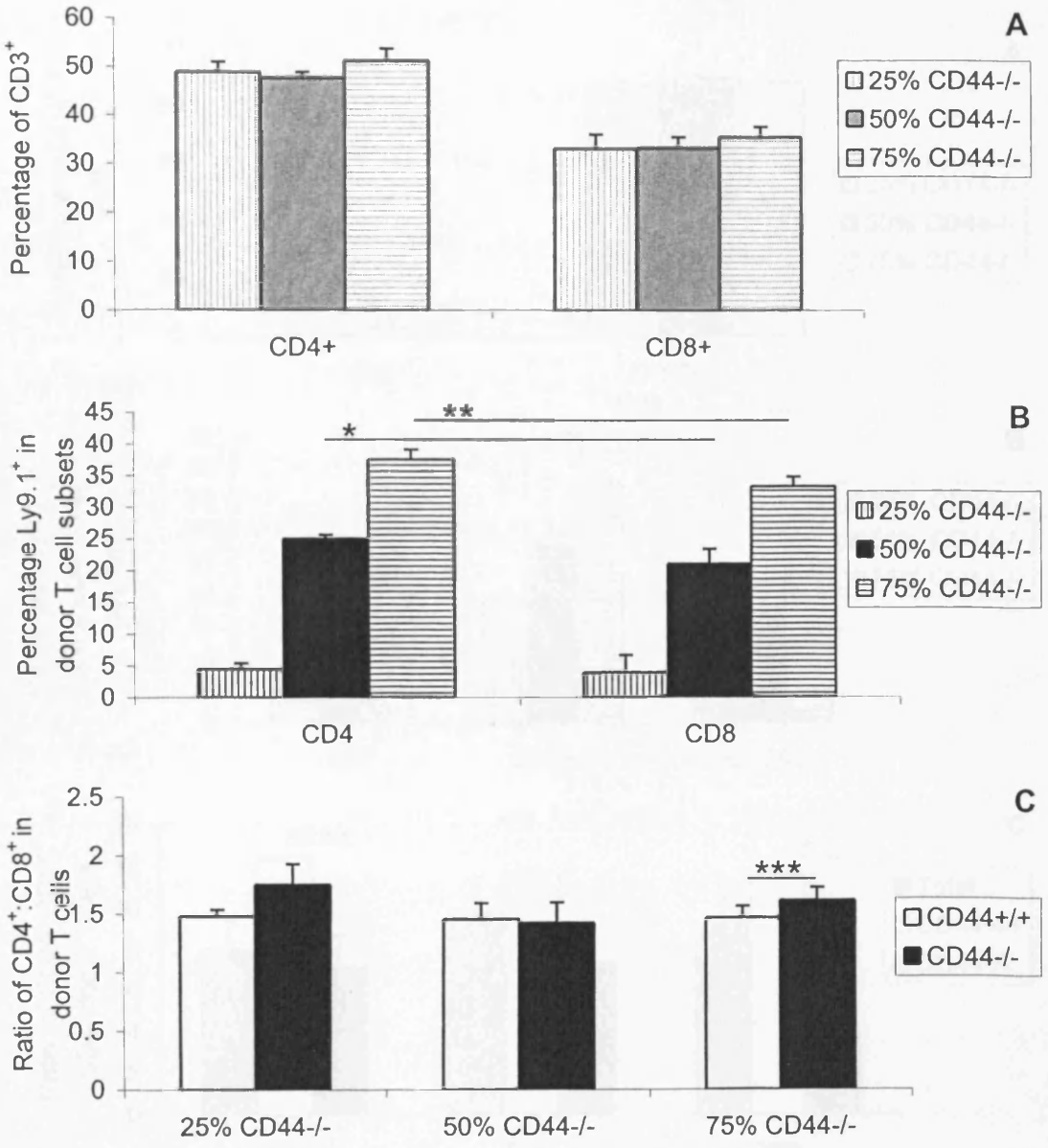

Figure 3.68. Analysis of T cell subsets in the lymph nodes of xCD44$^{+/+}$ & yCD44$^{-/-}$→ CD44$^{+/+}$ chimeras.

A. Percentages of CD4^{+} and CD8^{+} T cells in the lymph nodes of the various chimeras. B. The percentage of Ly9.1^{+} cells in each donor T cell lymph node subset per chimeric group. C. The CD4^{+}:CD8^{+} T cell ratio among CD44$^{-/-}$ and donor CD44$^{+/+}$ populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 8, 25% CD44$^{-/-}$, 1 group of 4, 50% CD44$^{-/-}$, 1 group of 6, 75% CD44$^{-/-}$. P values, 2-sample t test; *, P<0.05, **, P<0.01, ***, P<0.005

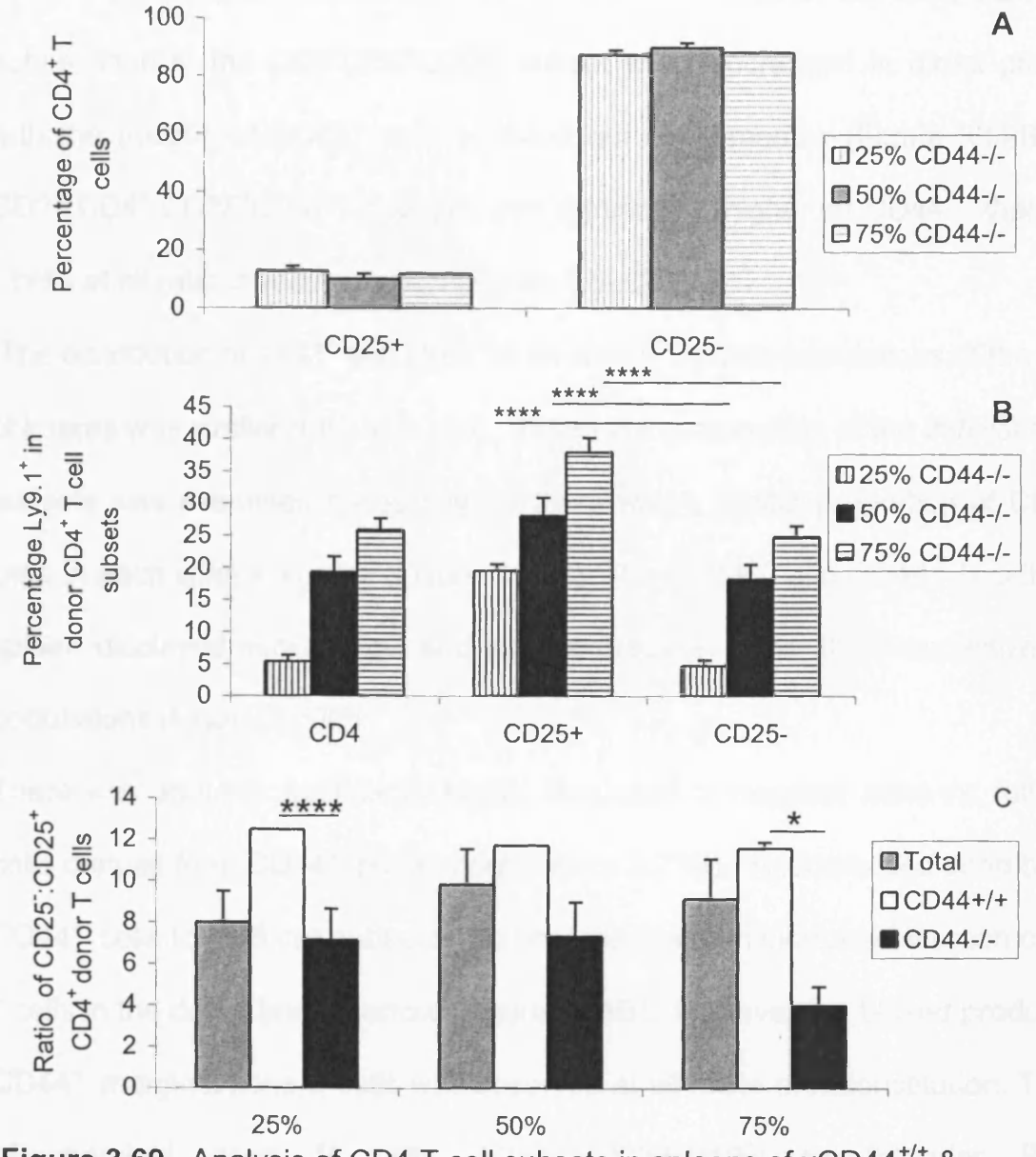

Figure 3.69. Analysis of CD4 T cell subsets in spleens of xCD44$^{+/+}$ & yCD44$^{-/-}$→ CD44$^{+/+}$ chimeras.

A. Percentages of CD25^{+} and CD25^{-} CD4^{+} T cells in spleens of the various chimeras. B. The percentage of Ly9.1^{+} cells in each donor CD4^{+} T cell subset per chimeric group. C. The CD25^{+}:CD25^{-} CD4^{+} T cell ratio among total, CD44$^{-/-}$ and donor CD44$^{+/+}$ populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 8, 25% CD44$^{-/-}$, 1 group of 4, 50% CD44$^{-/-}$, 1 group of 6, 75% CD44$^{-/-}$. P values, 2-sample t test; *, P<0.05, ****, P<0.001.

There was a higher contribution from the $CD44^{-/-}$ cells in the $CD3^+CD4^+CD25^+$ subset than in the $CD3^+CD4^+CD25^-$ subset which increased in direct proportion with the fraction of $CD44^{-/-}$ cells in the donor bone marrow (Figure 3.69B). The $CD25^-CD4^+$:$CD25^+CD4^+$ T cell ratio was significantly higher for $CD44^{+/+}$ than $CD44^{-/-}$ cells at all ratio of reconstitution (Figure 3.69C).

The distribution of $sIgM^+$ and $sIgD^+$ in the $CD19^+$ splenic populations of the various chimeras was similar (Figure 3.70A). When the composition of the individual B cell subsets was examined it was evident there was a similar proportion of $CD44^{-/-}$ B cells in each splenic subset (Figure 3.70B). The $CD44^{+/+}$ and $CD44^{-/-}$ B cells of the spleen displayed similar sIgD and sIgM expression within their respective $CD19^+$ populations (Figure 3.70C).

There was again a significantly higher frequency of marginal zone vs. follicular B cells derived from $CD44^{-/-}$ progenitors (Figure 3.71B). An increased contribution of $CD44^{-/-}$ cells to all B cell subsets was observed with an increased fraction of $CD44^{-/-}$ cells in the donor bone marrow (Figure 3.71B). However the biased production of $CD44^{-/-}$ marginal zone B cells was observed at all ratios of reconstitution. The ratio of marginal zone B cells ($CD21^+CD23^-CD19^+$) to follicular B cells ($CD21^+CD23^+CD19^+$) among $CD44^{+/+}$ cells decreased as the fraction of $CD44^{-/-}$ cells in the reconstitution increased (Figure 3.71C).

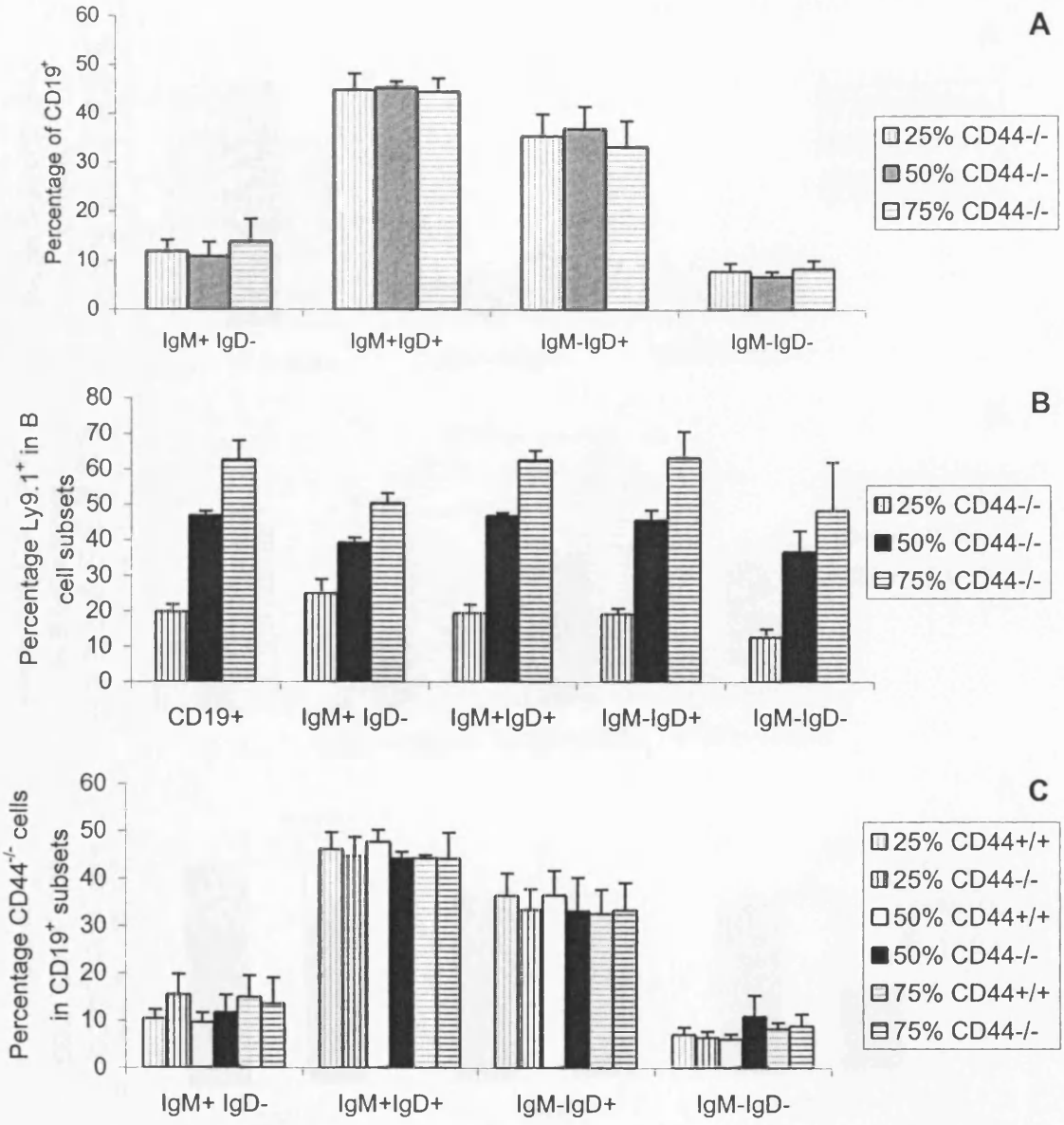

Figure 3.70. Analysis of B cell subsets in spleens of xCD44$^{+/+}$ & yCD44$^{-/-}$ → CD44$^{+/+}$ chimeras.

A. Percentages of splenic B cell (CD19^{+}) subsets defined by sIgM and sIgD expression in the various chimeras. B. The percentage of Ly9.1^{+} cells in each B cell subset. C. Distribution of sIgM and sIgD staining among CD44$^{-/-}$ or CD44$^{+/+}$ CD19^{+} cells. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 8, 25% CD44$^{-/-}$, 1 group of 4, 50% CD44$^{-/-}$, 1 group of 6, 75% CD44$^{-/-}$.

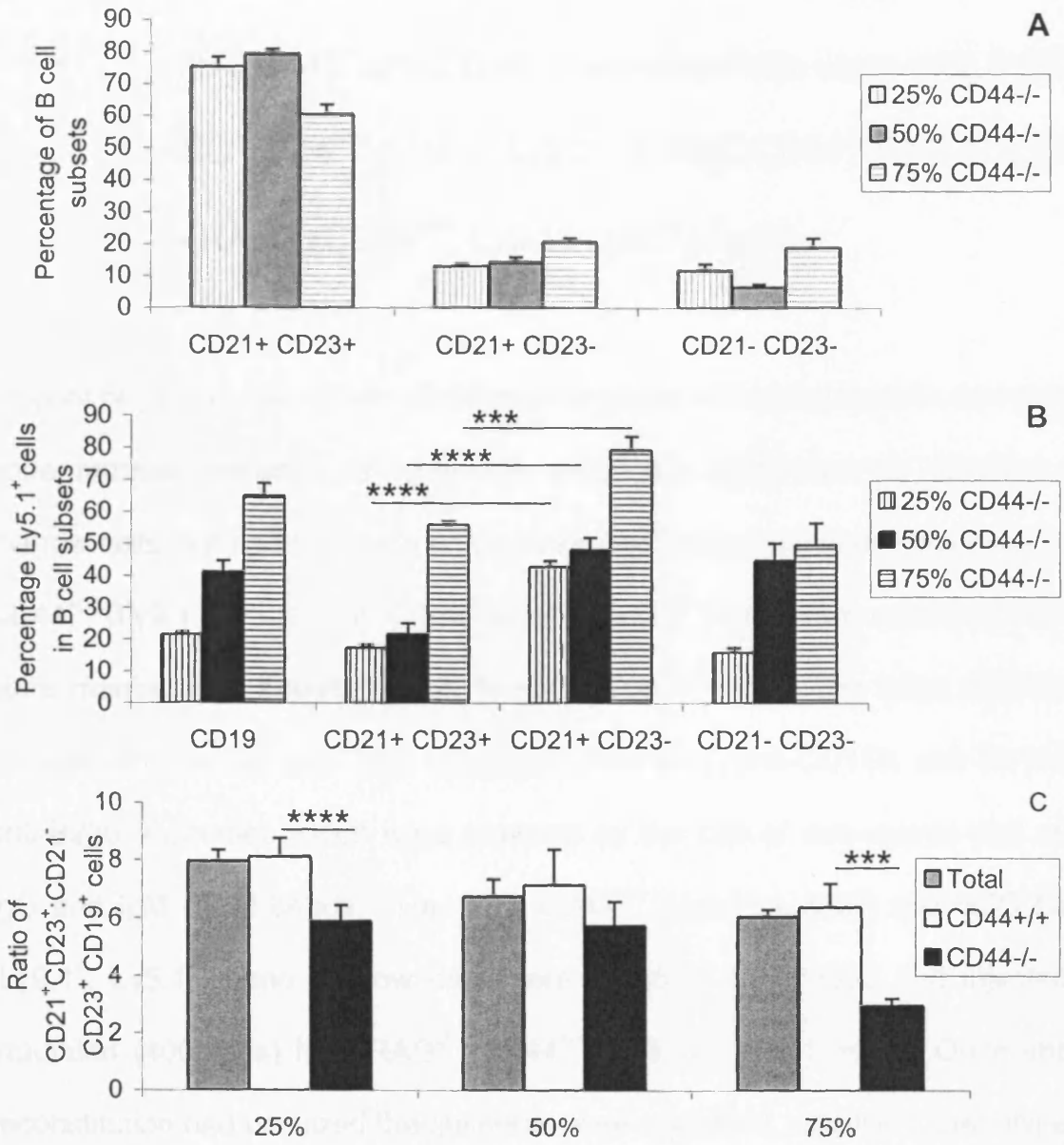

Figure 3.71. Analysis of follicular and marginal zone B cells in spleen of xCD44$^{+/+}$ & yCD44$^{-/-}$→ CD44$^{+/+}$ chimeras.

A. Percentages of splenic B cell (CD19^{+}) subsets defined by CD21 and CD23 in the spleens of the various chimeras. B. The percentage of Ly9.1^{+} (CD44$^{-/-}$) donor cells in each B cell subset in the various chimeras. C. Ratio of CD21^{+} CD23^{+}: CD21^{+} CD23^{-} cells among total, CD44$^{-/-}$ and CD44$^{+/+}$ CD19^{+} cells. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 8, 25% CD44$^{-/-}$, 1 group of 4, 50% CD44$^{-/-}$, 1 group of 6, 75% CD44$^{-/-}$. P values, 2-sample t test; ***, P<0.005, ****, P<0.001.

3.3.9. Phenotypic description of RAG host chimeras reconstituted with $CD44^{+/+}$ and $CD44^{-/-}$ haematopoietic stem cells (HSC): HSC $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & HSC $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) hosts

To control for possible effects of different numbers of haematopoietic precursors in bone marrow preparations, chimeras were also generated by injecting bone marrow cells that had first been enriched for haematopoietic stem cells (HSC). $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) and $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) mice were sacrificed and their bone marrow was depleted of all lineage positive cells. The bone marrow was labelled with purified anti-CD3, anti-Thy1.1, anti Gr-1, anti-CD11b, anti-Ter119 and anti-B220 antibodies which were removed by the use of anti-mouse and anti-rat IgG and IgM dynal beads. The HSC $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) and HSC $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) bone marrow cells were mixed in a 1:1 ratio and injected into irradiated (400 rads) host $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) mice. Once immune reconstitution had occurred the chimeras were sacrificed, and their phenotype was analysed by FACS. Cells derived from the $CD44^{-/-}$ progenitor cells were identified using an anti-Ly5.1 antibody.

These mice had normal cell numbers in their spleens and LN (appendix 1), and consistent percentages of total T and B cells, (Figures 3.72A, 3.73A).

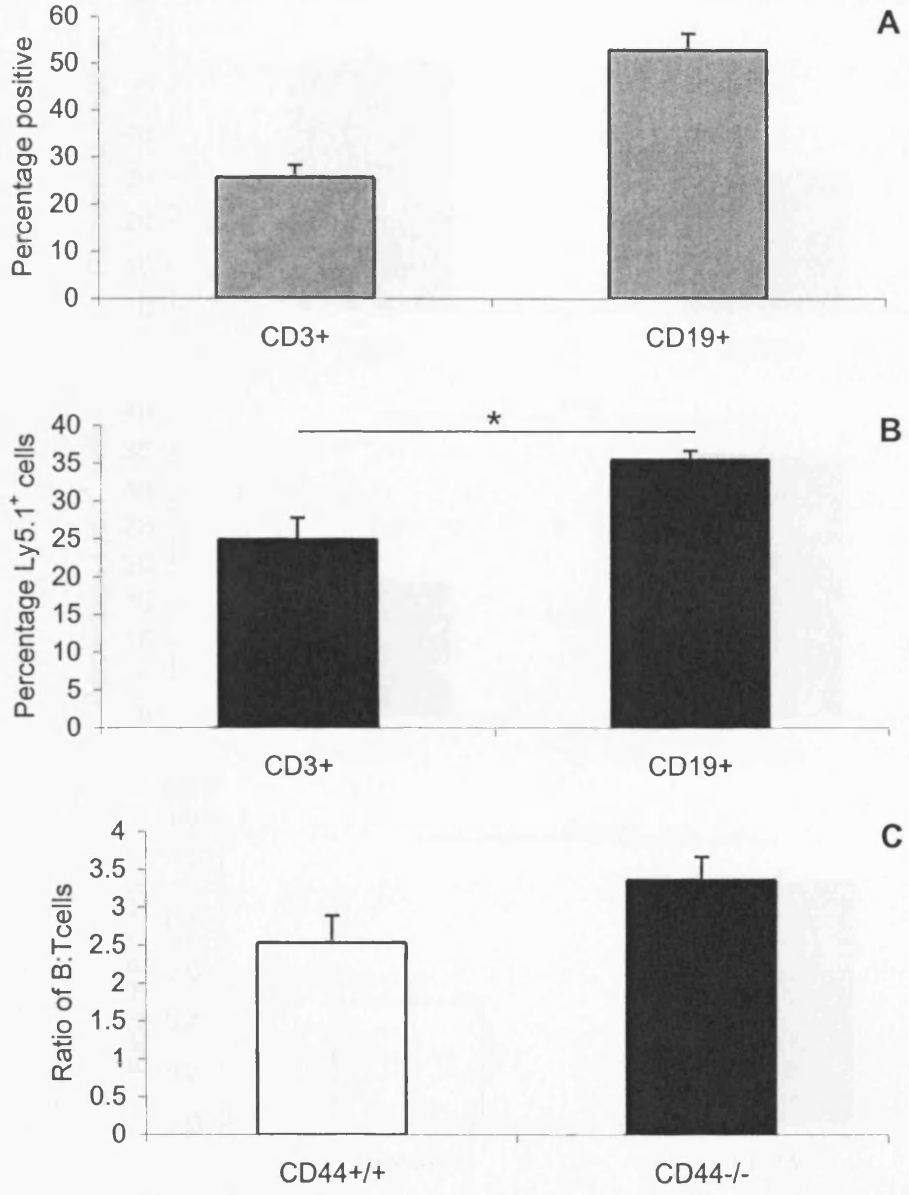

Figure 3.72. Analysis of T and B cells in spleens of HSC $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & HSC $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

A. Percentages of T and B cells in spleen. B. The percentage of $Ly5.1^{+}$ cells in each splenic population. C. The B:T cell ratio among $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 groups of 3. P values, 2-sample t test; *, $P<0.05$

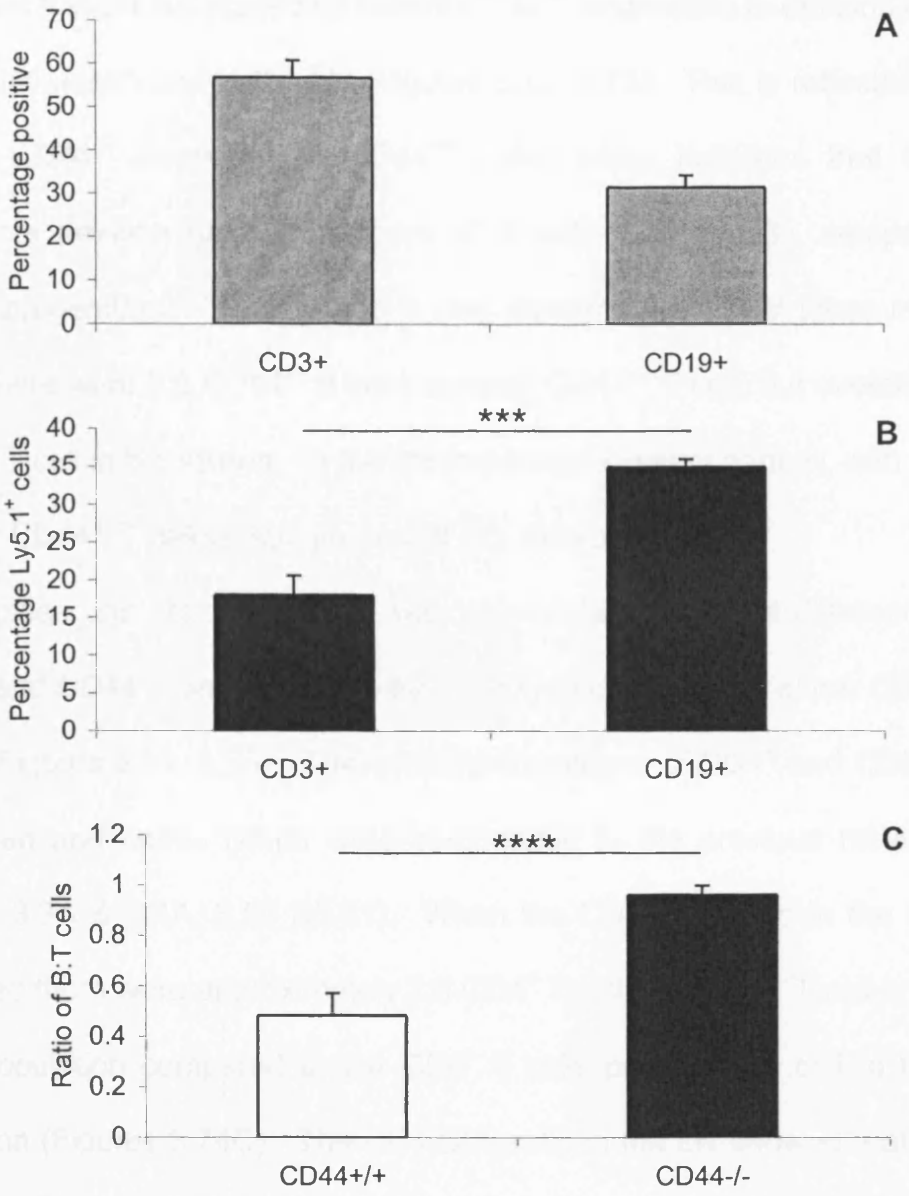

Figure 3.73. Analysis of T and B cell in lymph nodes of HSC CD44$^{+/+}$ (Ly9.1^{-}, Ly5.2^{+}) & HSC CD44$^{-/-}$ (Ly9.1^{+}, Ly5.1^{+}) → RAG$^{-/-}$ (CD44$^{+/+}$, Ly9.1^{-}, Ly5.1^{+}) chimeras.

A. Percentages of T and B cells in lymph nodes. B. The percentage of Ly9.1^{+} cells in each population. C. The B:T cell ratio among CD44$^{-/-}$ and CD44$^{+/+}$ donor populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 groups of 3. P values, 2-sample t test; ***, P<0.005, ****, P<0.001.

There was a slight but significant bias of $CD44^{-/-}$ progenitors to develop into B cells, in both the spleen and in the LN (Figures 3.72, 3.73). This is reflected in the B:T ratio of $CD44^{-/-}$ compared to $CD44^{+/+}$ cells, which indicates that the $CD44^{-/-}$ progenitors develop greater numbers of B cells than T cells, compared to the $CD44^{+/+}$ progenitors. The B:T ratio is also greater in the LN of these mice. In the spleen there were 3.5 $CD44^{-/-}$ B cells to every $CD44^{-/-}$ T cell, but overall 2.5 B cells to every T cell in the spleen. In the LN there was a similar pattern, with 1 $CD44^{-/-}$ B cells per $CD44^{-/-}$ T cell versus an overall B:T ratio of 0.4.

In the spleen and LN, again there was a small but significant difference between $CD44^{-/-}$ and $CD44^{+/+}$ progenitors in their ability to develop into either $CD4^+$ or $CD8^+$ T cells (Figures 3.74, 3.75). The overall percentages of $CD4^+$ and $CD8^+$ T cells in the spleen and nodes lymph were comparable to the previous mixed chimeras (Figures 3.3A & 3.4A, 3.50 &3.51). When the CD4:CD8 ratio in the spleen was calculated there were approximately 2.6 $CD4^+$ T cells per $CD8^+$ T cell in the $CD44^{-/-}$ T cell population compared to 2.4 $CD4^+$ T cells per $CD8^+$ T cell in the $CD44^{+/+}$ population (Figures 3.74C). The CD4:CD8 ratio in the LN showed that there were approximately 1.3 $CD4^+$ T cells per $CD8^+$ T cell in the $CD44^{-/-}$ T cell population compared to 1 $CD4^+$ T cells per $CD8^+$ T cell in the $CD44^{+/+}$ population (Figure 3.75C). The bias previously found in the $CD25^+CD4^+$ T cell population in the mixed chimeras (Figures 3.5& 3.6, 3.52 & 3.53), was again demonstrated in these HSC reconstituted RAG chimeras (Figures 3.76, 3.77). There was a significantly higher contribution from the $CD44^{-/-}$ cells in the $CD3^+CD4^+CD25^+$ subset than in the $CD3^+CD4^+CD25^-$ subset (Figure 3.76B, 3.77B).

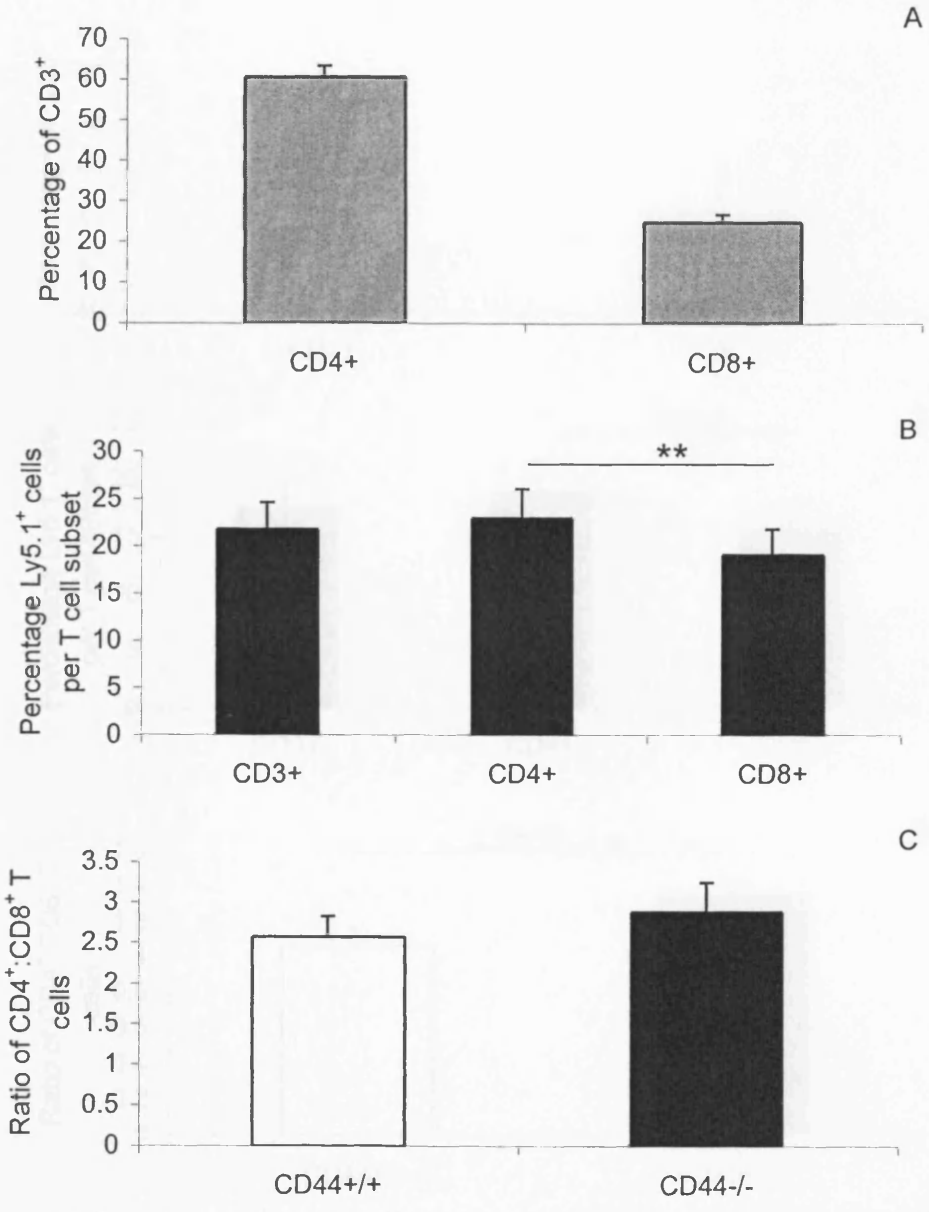

Figure 3.74. Analysis of T cell subsets in spleens of HSC $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & HSC $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) $\rightarrow$ $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

A. Percentages of $CD4^{+}$ and $CD8^{+}$ T cells in spleens. B. The percentage of $Ly9.1^{+}$ cells in each donor T cell population. C. The $CD4^{+}$:$CD8^{+}$ cell ratio among $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean $\pm$ s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 group of 3. P values, 2-sample t test; **, $P<0.01$

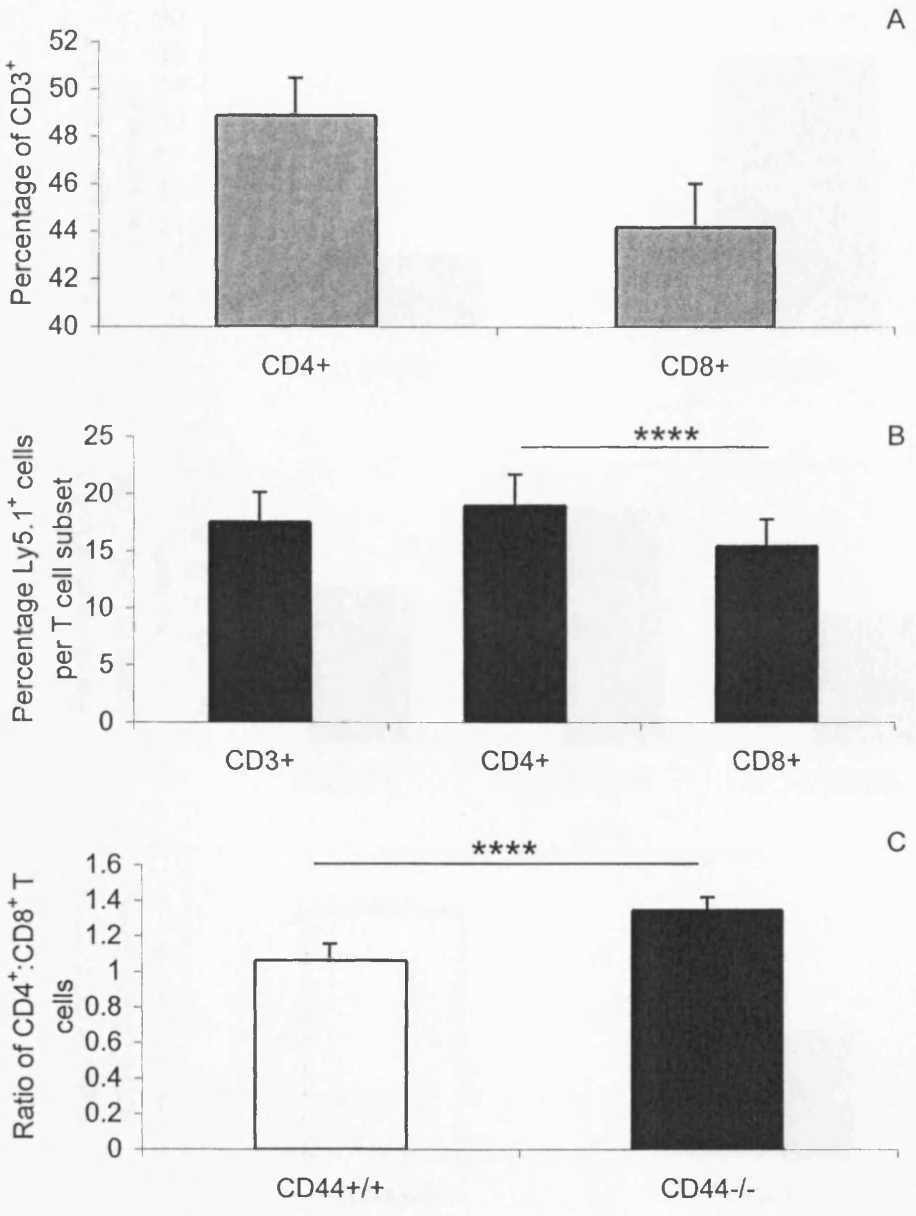

Figure 3.75. Analysis of T cell subsets in lymph nodes HSC $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & HSC $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) $\rightarrow$ $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

A. Percentages of $CD4^{+}$ and $CD8^{+}$ T cells in lymph nodes. B. The percentage of $Ly9.1^{+}$ cells in each donor T cell population. C. The $CD4^{+}$:$CD8^{+}$ cell ratio among $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean $\pm$ s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 groups of 3. P values, 2-sample t test; ****, $P<0.001$.

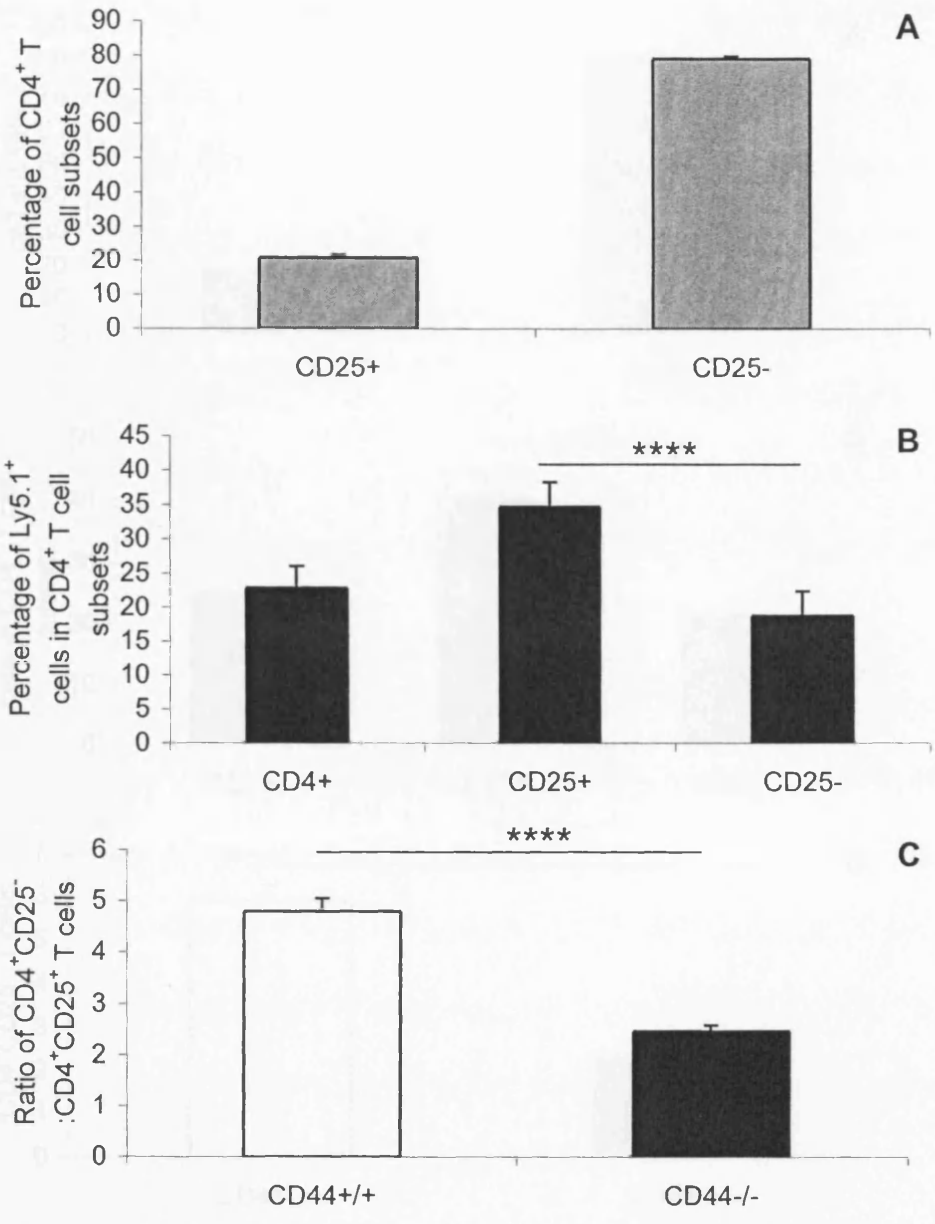

Figure 3.76. Analysis of CD4 T cell subsets in spleens of HSC $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & HSC $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

A. Percentages of $CD25^{+}$ and $CD25^{-}$ $CD4^{+}$ T cells in spleens. B. The percentage of $Ly9.1^{+}$ cells in each donor $CD4^{+}$ T cell subset. C. The $CD4^{+}CD25^{-}$:$CD4^{+}CD25^{+}$ T cell ratio among $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 groups of 3. P values, 2-sample t test; ****, $P<0.001$.

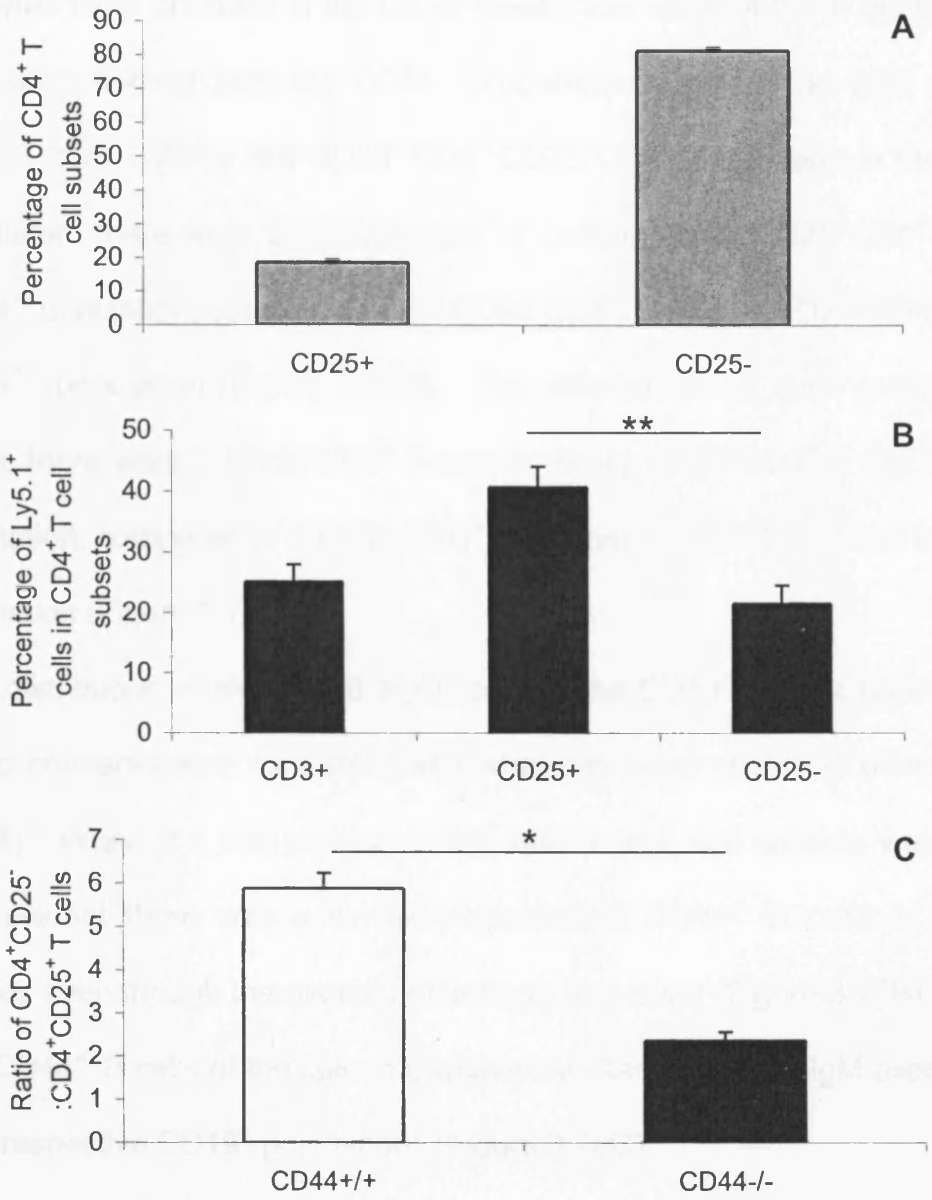

Figure 3.77. Analysis of $CD4^+$ T cell subsets in lymph nodes of HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras.

A. Percentages of $CD25^+$ and $CD25^-$ $CD4^+$ T cells in lymph nodes. B. The percentage of $Ly9.1^+$ cells in each donor $CD4^+$ T cell population. C. The $CD4^+CD25^-$:$CD4^+CD25^+$ T cell ratio among $CD44^{-/-}$ and $CD44^{+/+}$ donor populations. The data are mean ± s.e.m. obtained from 1 group of 2 chimeras. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$

This was most dramatic in the LN of these mice where 40% of the $CD25^+CD4^+$ T cells were derived from the $CD44^{-/-}$ progenitors compared to 20% of the $CD25^-$ $CD4^+$ T cells. When the $CD25^-$ $CD4^+$:$CD25^+CD4^+$ T cell ratio in the spleen was calculated, there were 2.5 $CD25^-CD4^+$ T cells to every $CD25^+CD4^+$ T cell in the $CD44^{-/-}$ population, compared to 4.5 $CD25^-CD4^+$ T cell per $CD25^+CD4^+$ T cell in the $CD44^{+/+}$ population (Figure 3.76C). This difference was even greater in the LN, where there were 2 $CD25^-CD4^+$ T cells to every $CD25^+CD4^+$ T cell in the $CD44^{-/-}$ population, compared to 6 $CD25^-CD4^+$ T cell per $CD25^+CD4^+$ T cell in the $CD44^{+/+}$ population (Figure 3.77C).

The distribution of $sIgM^+$ and $sIgD^+$ cells in the $CD19^+$ splenic populations of the mixed chimeras was consistent with what has been observed previously (Figure 3.78A). When the composition of the individual B cell subsets was examined it was evident there was a similar proportion of $CD44^{-/-}$ B cells in each splenic subset, even though the overall percentage was lower (Figure 3.78B). The $CD44^{+/+}$ and $CD44^{-/-}$ B cells of the spleen displayed similar sIgD and sIgM expression within their respective $CD19^+$ populations (Figure 3.78C).

There was a significant difference in the percentage of marginal zone B cells vs. follicular B cells derived from $CD44^{-/-}$ progenitors (Figure 3.79). There was an increase in the percentage of $CD44^{-/-}$ cells present among the marginal zone B cells ($CD19^+CD21^+CD23^-$) compared to the amount found in the follicular B cell population ($CD19^+CD21^+CD23^+$) (Figure 3.79B). This observation is reinforced by a higher marginal zone frequency in the $CD44^{-/-}$ B cell population.

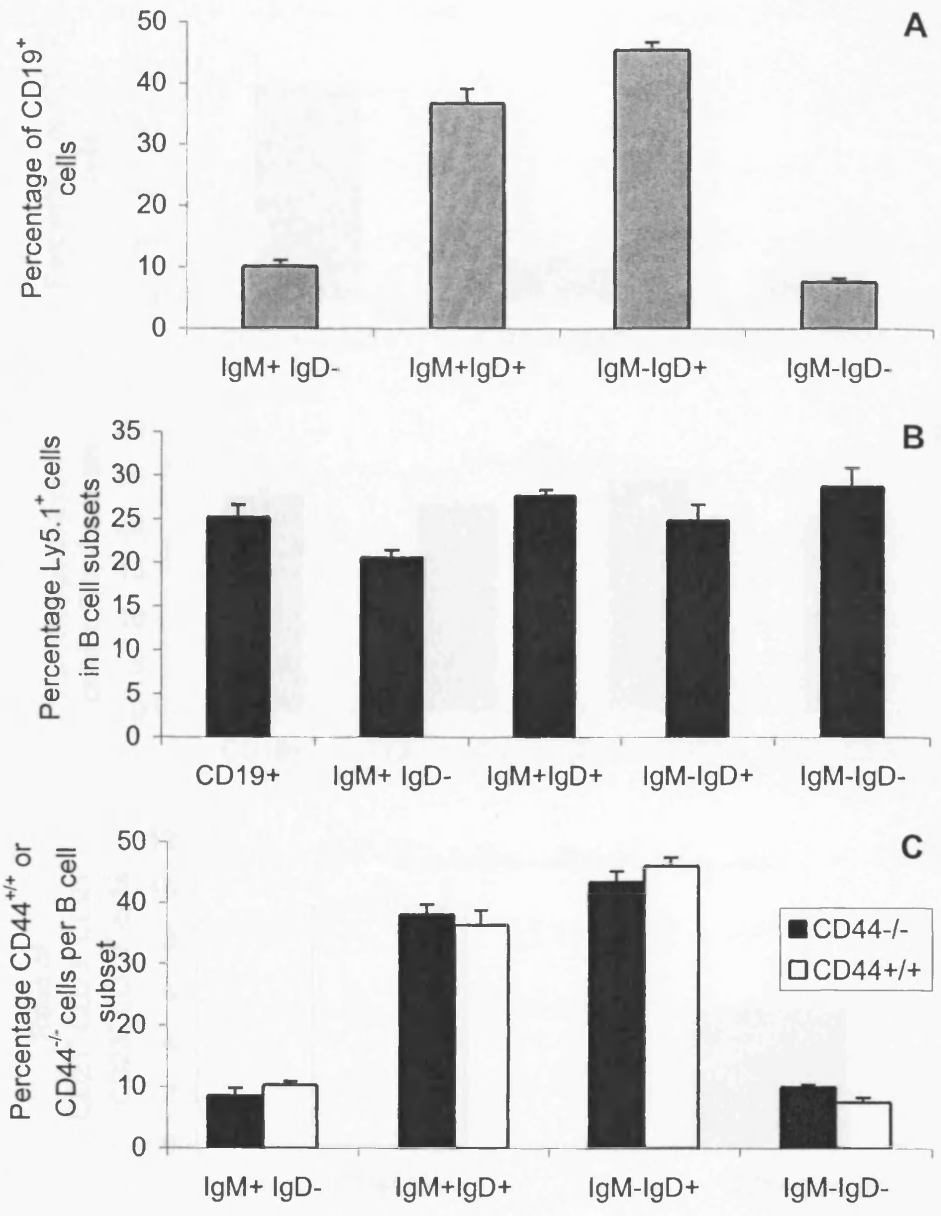

Figure 3.78. Analysis of B cell subsets in spleens of HSC $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & HSC $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

A. Percentages of splenic B cell ($CD19^{+}$) subsets defined by sIgM and sIgD expression. B. The percentage of $Ly9.1^{+}$ cells in each B cell subset. C. Distribution of sIgM and sIgD staining among $CD44^{-/-}$ or $CD44^{+/+}$ $CD19^{+}$ cells. Data are mean ± s.e.m. obtained from 1 group of 2 and 2 groups of 3 mice.

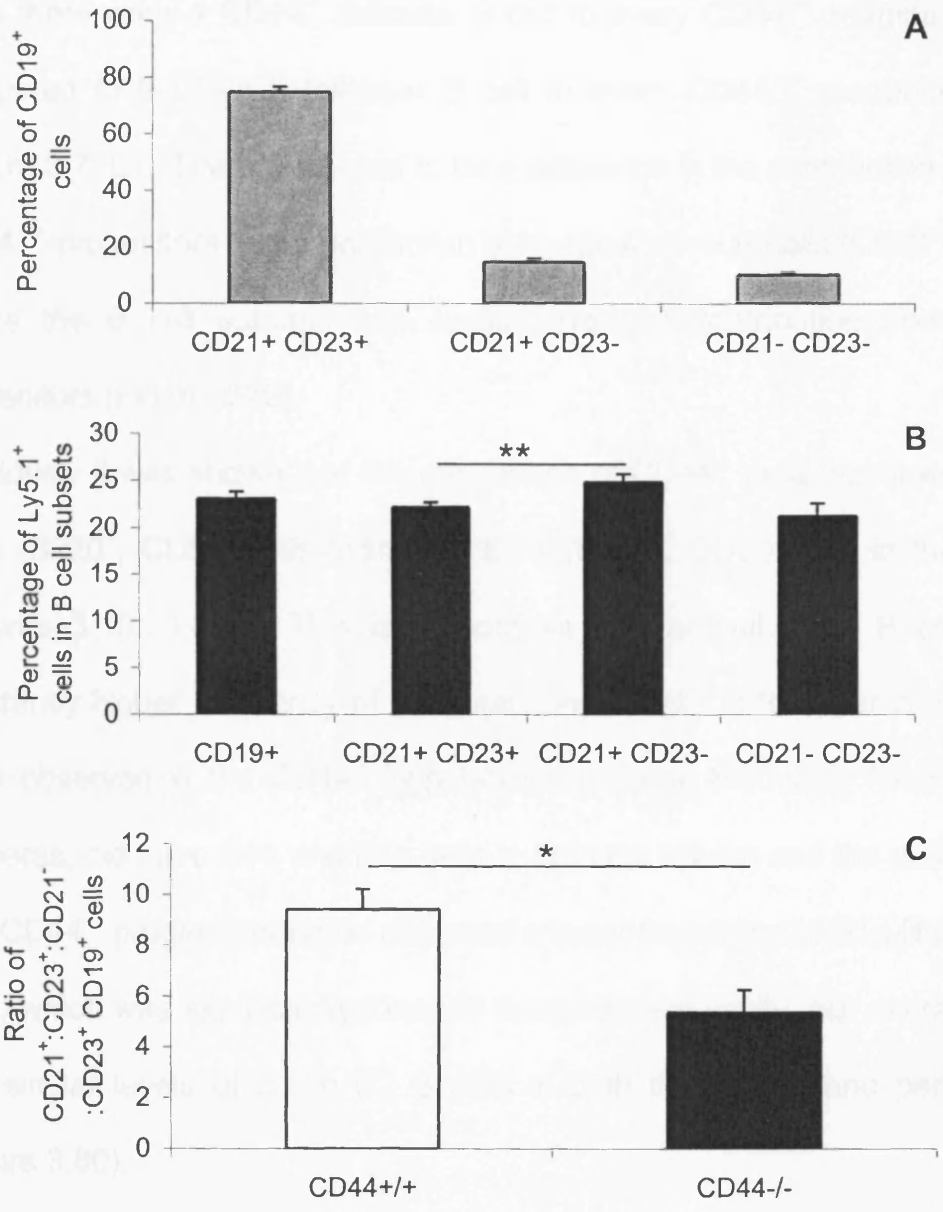

Figure 3.79. Analysis of follicular and marginal zone B cells in spleen of HSC CD44$^{+/+}$ (Ly9.1^{-}, Ly5.2^{+}) & HSC CD44$^{-/-}$ (Ly9.1^{+}, Ly5.1^{+}) → RAG$^{-/-}$ (CD44$^{+/+}$, Ly9.1^{-}, Ly5.1^{+}) chimeras.

A. Percentages of splenic B cell (CD19^{+}) subsets defined by CD21 and CD23. B. The percentage of Ly9.1^{+} (CD44$^{-/-}$) donor cells in each B cell subset. C. Ratio of CD21^{+} CD23^{+}: CD21^{+} CD23^{-} cells among total, CD44$^{-/-}$ and CD44$^{+/+}$ CD19^{+} cells. The data are mean ± s.e.m. obtained from 3 groups of chimeras: 1 group of 2 and 2 groups of 3. P values, 2-sample t test; *, P<0.05

Thus there were 4 $CD44^{-/-}$ follicular B cell to every $CD44^{-/-}$ marginal zone B cell, compared to 9 $CD44^{+/+}$ follicular B cell to every $CD44^{+/+}$ marginal zone B cell (Figure 3.79C). There appeared to be a difference in the contribution of $CD44^{-/-}$ vs. $CD44^{+/+}$ progenitors to the production of marginal zone B cells ($CD21^{+}CD23^{-}B220^{+}$) where the B cell subsets had similar levels of contribution from the $CD44^{-/-}$ progenitors (Figure 3.79).

Previously it was shown that the percentage of $CD44^{-/-}$ cells was lower among the B-1a ($B220^{+}$, $CD5^{+}$) cells than non B-1a ($B220^{+}$, $CD5^{-}$) cells in the peritoneum (Figures 3.10, 3.80). This is in contrast to marginal zone B cells, where a constantly higher frequency of marginal zone B cells to follicular zone B cells has been observed in the $CD44^{-/-}$ populations. These HSC only $RAG^{-/-}$ host mixed chimeras, did have B1a and B1b cells in both the spleen and the peritoneal cavity. The $CD44^{-/-}$ progenitors again produced a lower frequency of B1a-B cells to B1b-B cells, which was significantly lower in the peritoneal cavity, but overall had similar had similar levels of B1 to B2 B cells in both the spleen and peritoneal cavity (Figure 3.80).

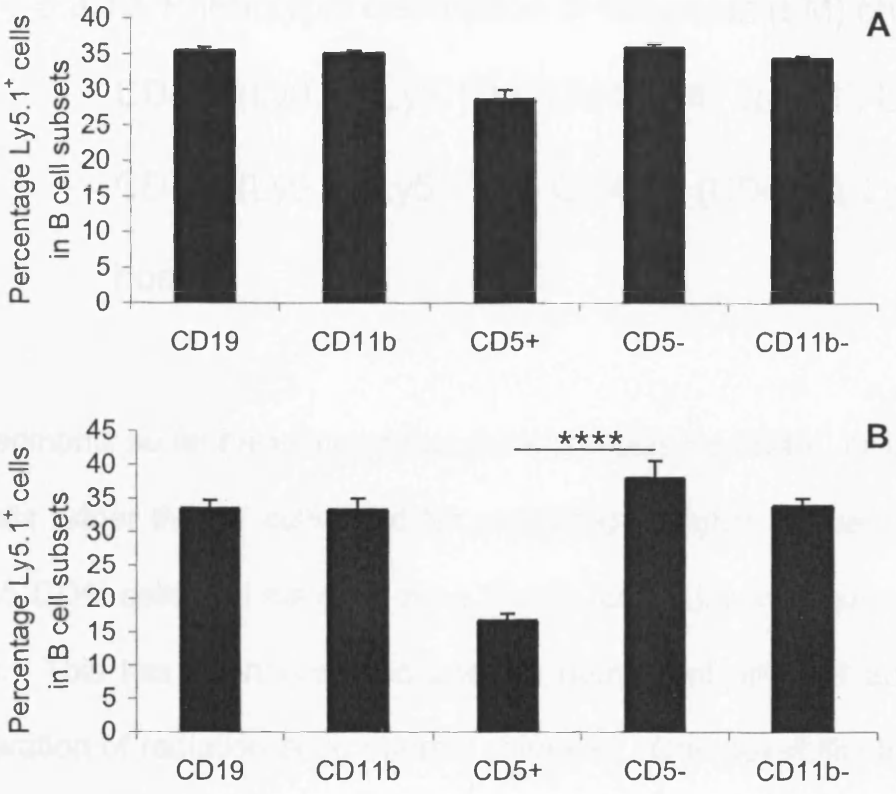

Figure 3.80. Analysis of B1 and B2 cells in spleen and peritoneal cavity of HSC CD44$^{+/+}$ (Ly9.1^{-}, Ly5.2^{+}) & HSC CD44$^{-/-}$ (Ly9.1^{+}, Ly5.1^{+}) → RAG$^{-/-}$ (CD44$^{+/+}$, Ly9.1^{-}, Ly5.1^{+}) chimeras.

A. Percentage of Ly5.1^{+} cells in each B cell (CD19^{+}) subset of spleen. B. Percentage of Ly5.1^{+} cells in each B cell (CD19^{+}) subset of peritoneal cavity. Data are mean ± s.e.m. obtained from 2 groups of 3 mice. P values, 2-sample t test; ****, P<0.001.

3.3.10. Phenotypic description of littermate (LM) chimeras: LM $CD44^+$ ($Ly9.1^+$, $Ly5.1^+$) & LM $CD44^{-/-}$ ($Ly9.1^-$, $Ly5.1^+$) Or LM $CD44^+$ ($Ly9.1^-$, $Ly5.1^+$) → $CD44^{+/+}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.2^+$) hosts.

Experiments so far have shown that there is a bias for $CD44^{-/-}$ cells developing into B cells rather than T cells, and for producing a higher frequency of $CD4^+$ cells, $CD25^+CD4^+$ cells and marginal zone B cells, and a lower frequency of $CD5^+$ B1 B cells. This has been observed using a number of different approaches in the generation of radiation bone marrow chimeras. One possibility that has not been investigated is that differences in background genes, rather than CD44, accounted for the differential production of lymphocytes by different donor populations. This was a concern because the $CD44^{-/-}$ mice are on a mixed background (129Sv X C57Bl/6). Therefore it was decided to backcross these mice to C57Bl/6 mice, and use the $CD44^+$ and $CD44^-$ offspring (littermates) to generate mixed bone marrow chimeras.

Female $CD44^{-/-}$ mice were crossed with a male C57Bl/6 mouse, the resulting offspring were then mated. The offspring of this cross were screened by tail bleeding, for Ly9.1 and CD44 expression. The correct combination of littermate (LM) mice were then sacrificed (LM $CD44^+$, $Ly9.1^+$), (LM $CD44^{-/-}$, $Ly9.1^-$) & (LM $CD44^+$, $Ly9.1^-$) their bone marrow T cell depleted by antibody mediated complement cytotoxicity. The LM $CD44^+$ ($Ly9.1^+$) and LM $CD44^{-/-}$ ($Ly9.1^-$) or as a control the LM $CD44^+$ ($Ly9.1^+$) and LM $CD44^+$ ($Ly9.1^-$) bone marrow cells were

mixed in a 1:1 ratio and injected into irradiated (900 rads) host $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) mice. Once immune reconstitution had occurred the mice were sacrificed, and their phenotype was analysed by FACS. Cells derived from the donor progenitor cells were identified using an anti-Ly9.1 (CD229.1) antibody in conjunction with an anti-Ly5.1 antibody. B cells were identified using the anti-Ly9.1 antibody alone due to lack of radioresistant B cells. Donor T cells were identified using the anti-Ly9.1 antibody and anti-Ly5.1 of anti-Ly5.2 antibodies.

These chimeric mice had normal cell numbers in their spleens and LN (appendix 1), and consistent percentages of total T and B cells (Figures 3.81 A & B, 3.82 A & B). The bias of $CD44^{-/-}$ progenitors to develop into B cells rather than T cells in chimeras generated with mixed $CD44^{+}Ly9.1^{+}$ and $CD44^{-/-}Ly9.1^{-}$ bone marrow cells was again significant in both the spleen and LN (Figures 3.81C & E and 3.82C & E). Notably, however differential production of T and B cells by the two donor populations was also observed in the control chimeras. Thus, although both donor populations were $CD44^{+}$, the $Ly9.1^{+}$ cells produced a higher proportion of B cells than the $Ly9.1^{-}$ cells (Figures 3.81D and 3.82D).

In the spleen of experimental chimeras, there was a significant difference in the ability of $CD44^{-/-}$ progenitors to develop into $CD4^{+}$ rather than $CD8^{+}$ T cells with a higher CD4:CD8 ratio for the $CD44^{-}$ than $CD44^{+}$ cells as observed before (Figures 3.83C & E). This was also seen in LN (Figure 3.84E), while significant differences in CD4:CD8 ratio between $Ly9.1^{+}$ and $Ly9.1^{-}$ cells was not observed in the control chimeras (Figures 3.83F & 3.84 F)

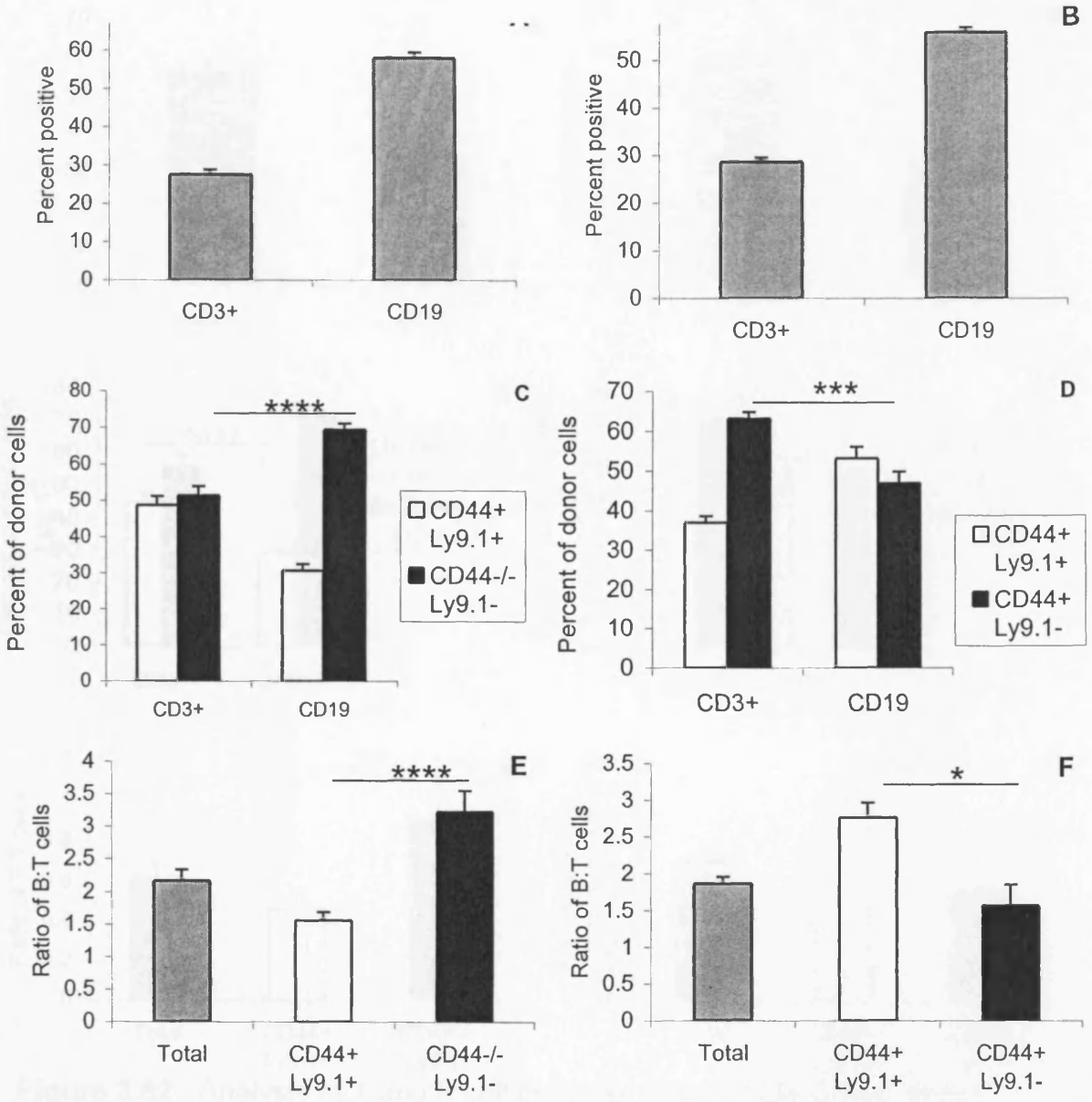

Figure 3.81. Analysis of T and B cells in spleens of LM CD44^{+} (Ly9.1^{+}, Ly5.1^{+}) & LM CD44$^{-/-}$ (Ly9.1^{-}, Ly5.1^{+}) / Or LM CD44^{+} (Ly9.1^{+}, Ly5.1^{+}) & LM CD44^{+} (Ly9.1^{-}, Ly5.1^{+}) & $\rightarrow$ CD44$^{+/+}$ (CD44$^{+/+}$, Ly9.1^{-}, Ly5.2^{+}) chimeras.

A, B. Percentages of T and B cells in spleen. C, D. The percentage of Ly9.1^{+} and Ly9.1^{-} cells in each splenic population. E, F. The B:T cell ratio among total, and donor Ly9.1^{-} and Ly9.1^{+} populations. A, C, E, are LM CD44^{+} & LM CD44$^{-/-}$ $\rightarrow$ CD44$^{+/+}$ hosts, and B, D, F, are LM CD44^{+} & LM CD44^{+} $\rightarrow$ CD44$^{+/+}$ hosts. The data are mean $\pm$ s.e.m. obtained from 2 groups of chimeras: 1 group of 8 and 1 group of 9. P values, 2-sample t test; *, $P<0.05$, ***, $P<0.005$, ****, $P<0.001$.

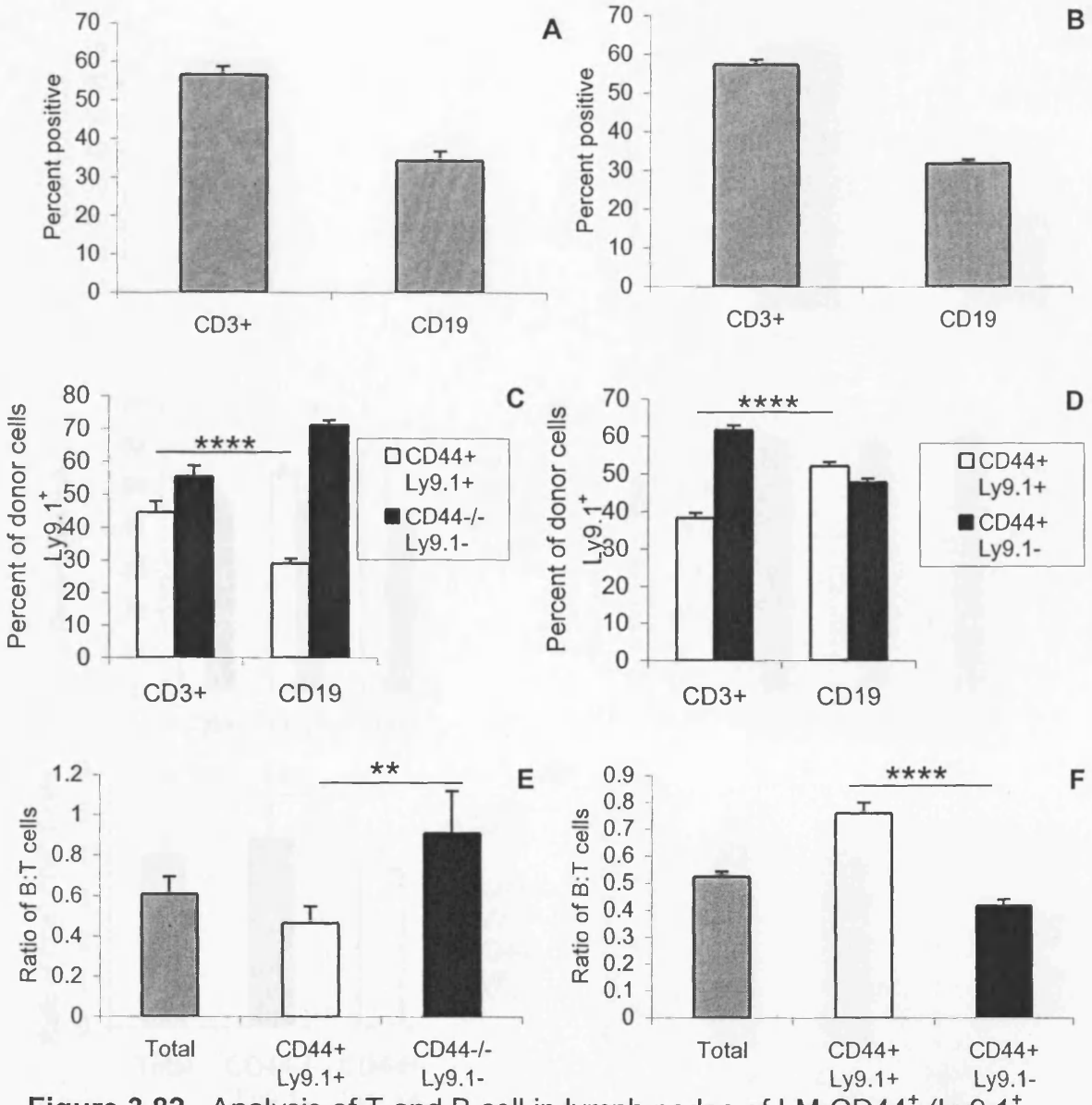

Figure 3.82. Analysis of T and B cell in lymph nodes of LM $CD44^{+}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) & LM $CD44^{-/-}$ ($Ly9.1^{-}$, $Ly5.1^{+}$) / Or LM $CD44^{+}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) & LM $CD44^{+}$ ($Ly9.1^{-}$, $Ly5.1^{+}$) & $\rightarrow$ $CD44^{+/+}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.2^{+}$) chimeras.

A, B. Percentages of T and B cells in lymph nodes. C, D. The percentage of $Ly9.1^{+}$ and $Ly9.1^{-}$ cells in each population. E, F. The B:T cell ratio among donor of $Ly9.1^{+}$ and $Ly9.1^{-}$ populations. A, C, E, are LM $CD44^{+}$ & LM $CD44^{-/-}$ $\rightarrow$ $CD44^{+/+}$ hosts, and B, D, F, are LM $CD44^{+}$ & LM $CD44^{+}$ $\rightarrow$ $CD44^{+/+}$ hosts. The data are mean $\pm$ s.e.m. obtained from 2 groups of chimeras: 1 group of 8 and 1 group of 9. P values, 2-sample t test; **, $P<0.01$, ****, $P<0.001$.

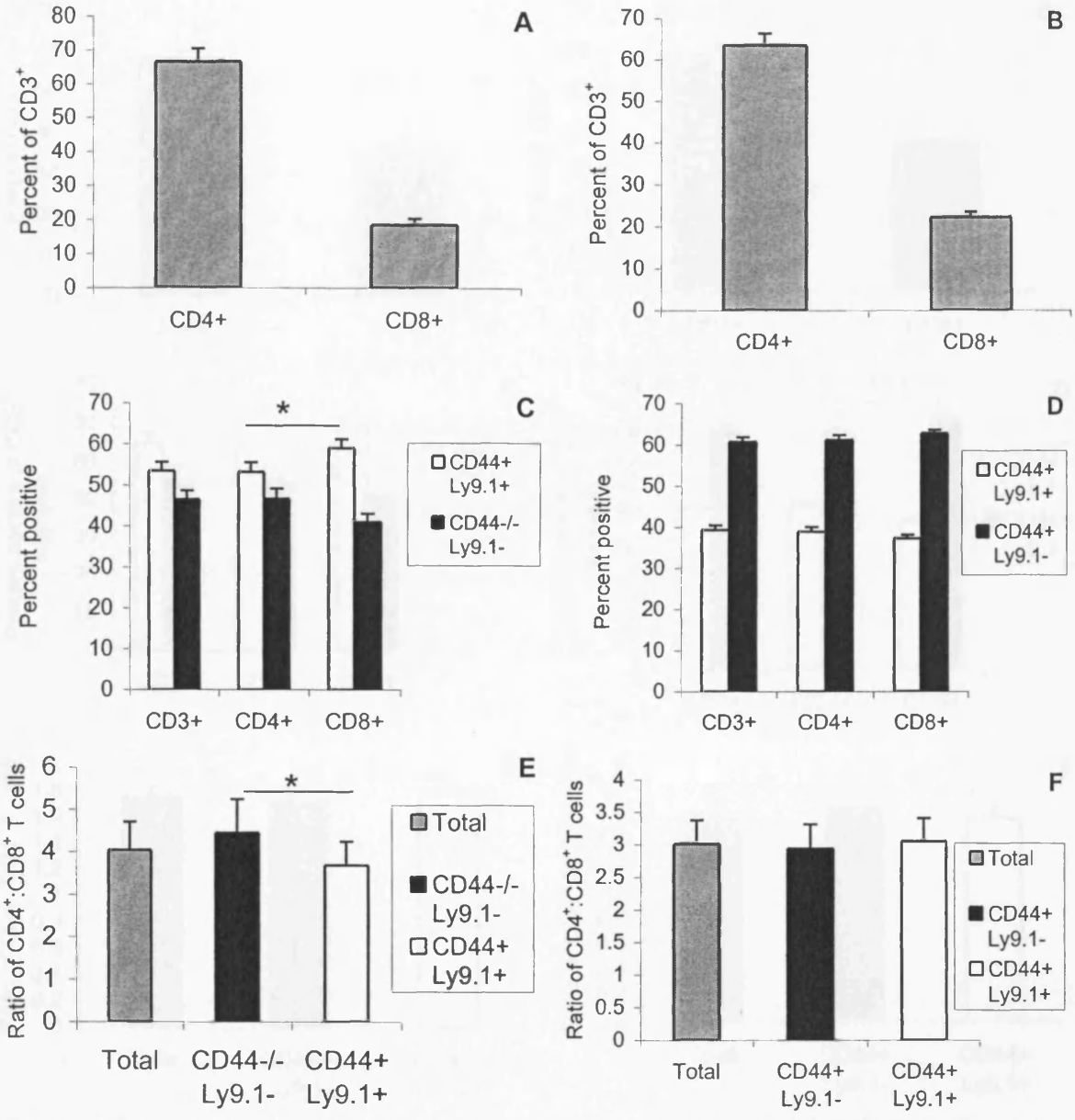

Figure 3.83. Analysis of T cell subsets spleens of LM CD44$^+$ (Ly9.1$^+$, Ly5.1$^+$) & LM CD44$^{-/-}$ (Ly9.1$^-$, Ly5.1$^+$) / Or LM CD44$^+$ (Ly9.1$^+$, Ly5.1$^+$) & LM CD44$^+$ (Ly9.1$^-$, Ly5.1$^+$) & $\rightarrow$ CD44$^{+/+}$ (CD44$^{+/+}$, Ly9.1$^-$, Ly5.2$^+$) chimeras.

A, B. Percentages of CD4$^+$ and CD8$^+$ T cells in spleens. C, D. The percentage of Ly9.1$^+$ and Ly9.1$^-$ cells in each donor T cell population. E, F. The CD4$^+$:CD8$^+$ cell ratio among total, and donor Ly9.1$^+$ and Ly9.1$^-$ populations. A, C, D, are LM CD44$^+$ & LM CD44$^{-/-}$ $\rightarrow$ CD44$^{+/+}$ hosts, and B, D, F, are LM CD44$^+$ & LM CD44$^+$ $\rightarrow$ CD44$^{+/+}$ hosts. The data are mean $\pm$ s.e.m. obtained from 2 groups of chimeras: 1 group of 8 and 1 group of 9. P values, 2-sample t test; *, P<0.05.

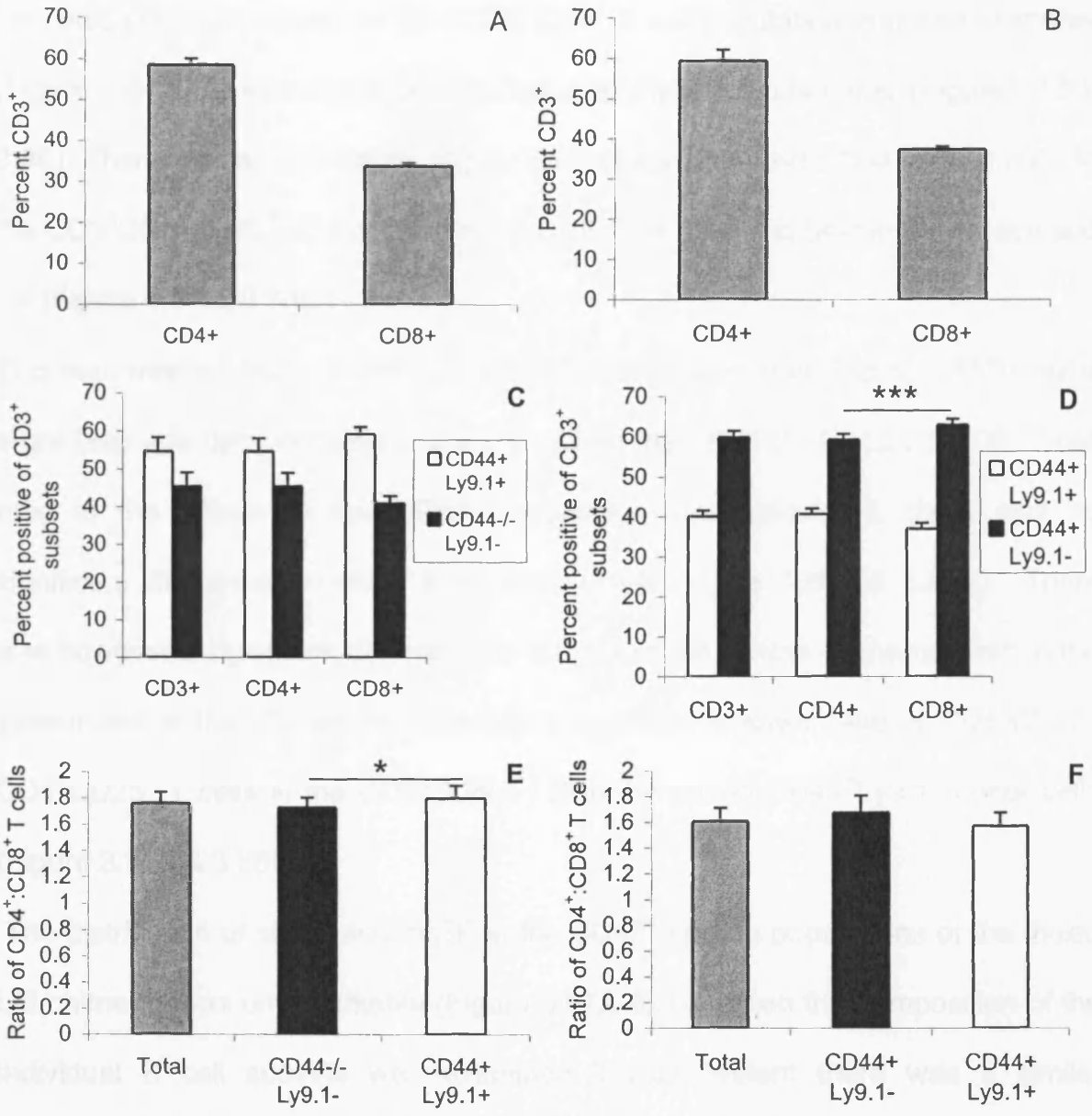

Figure 3.84. Analysis of T cell subsets in lymph nodes of LM CD44$^+$ (Ly9.1$^+$, Ly5.1$^+$) & LM CD44$^{-/-}$ (Ly9.1$^-$, Ly5.1$^+$) / Or LM CD44$^+$ (Ly9.1$^+$, Ly5.1$^+$) & LM CD44$^+$ (Ly9.1$^-$, Ly5.1$^+$) & $\rightarrow$ CD44$^{+/+}$ (CD44$^{+/+}$, Ly9.1$^-$, Ly5.2$^+$) chimeras.

A, B. Percentages of CD4$^+$ and CD8$^+$ T cells in lymph nodes. C, D. The percentage of Ly9.1$^+$ and Ly9.1$^-$ cells in each donor T cell population. E, F. The CD4$^+$:CD8$^+$ cell ratio among total, and donor Ly9.1$^+$ and Ly9.1$^-$ populations. A, C, E, are LM CD44$^+$ & LM CD44$^{-/-}$ $\rightarrow$ CD44$^{+/+}$ hosts, and B, D, F, are LM CD44$^+$ & LM CD44$^+$ $\rightarrow$ CD44$^{+/+}$ hosts. The data are mean $\pm$ s.e.m. obtained from 2 groups of chimeras: 1 group of 8 and 1 group of 9. P values, 2-sample t test; *, P<0.05, ***, P<0.001.

The bias previously found in the $CD25^+CD4^+$ T cell population in mixed chimeras (Figure 3.5, 3.6), was again demonstrated in these LM chimeras (Figures 3.85, 3.86). There was a significantly higher contribution from the $CD44^{-/-}$ $Ly9.1^-$cells to the $CD3^+CD4^+CD25^+$ subset than in the $CD3^+CD4^+CD25^-$ subset in the spleen and LN (Figure 3.85C, 3.86C).

This bias was not found in the spleen of the control chimeras (Figure 3.85D), but a slight bias was demonstrated in the LN. When the $CD25^-CD4^+$: $CD25^+CD4^+$ T cell ratio in the spleen of the $CD44^{-/-}$ population was calculated, there was no significant differences in either the spleen or LN (Figure 3.85E & 3.86E). There was however a significant difference in the ratio in the control chimeras, both in the spleen and in the LN where there was a significantly lower ratio of $CD4^+CD25^-$: $CD4^+CD25^+$ T cells in the $CD44^+Ly9.1^-$, than the paired $CD44^+Ly9.1^+$ donor cells (Figure 3.85F & 3.86F).

The distribution of $sIgM^+$ and $sIgD^+$ in the $CD19^+$ splenic populations of the mixed LM chimeras was unremarkable (Figure 3.87A & B). When the composition of the individual B cell subsets was examined it was evident there was a similar proportion of $CD44^{-/-}$ and $CD44^+$ B cells in each splenic subset (Figure 3.87C & D). The $CD44^{+/+}$ and $CD44^{-/-}$ B cells of the spleen displayed similar sIgD and sIgM expression within their respective $CD19^+$ populations (Figure 3.54E & F).

There was no significant differences in the contribution of $CD44^{-/-}$ vs. $CD44^+$ progenitors or $Ly9.1^+$ vs. $Ly9.1^-$ in the production of marginal zone B cells ($CD21^+CD23^-B220^+$) (Figure 3.88C & D), or in the ratio of marginal zone B cells ($CD21^+CD23^-CD19^+$) and follicular B cells ($CD21^+CD23^+CD19^+$), (Figure 3.88E & F).

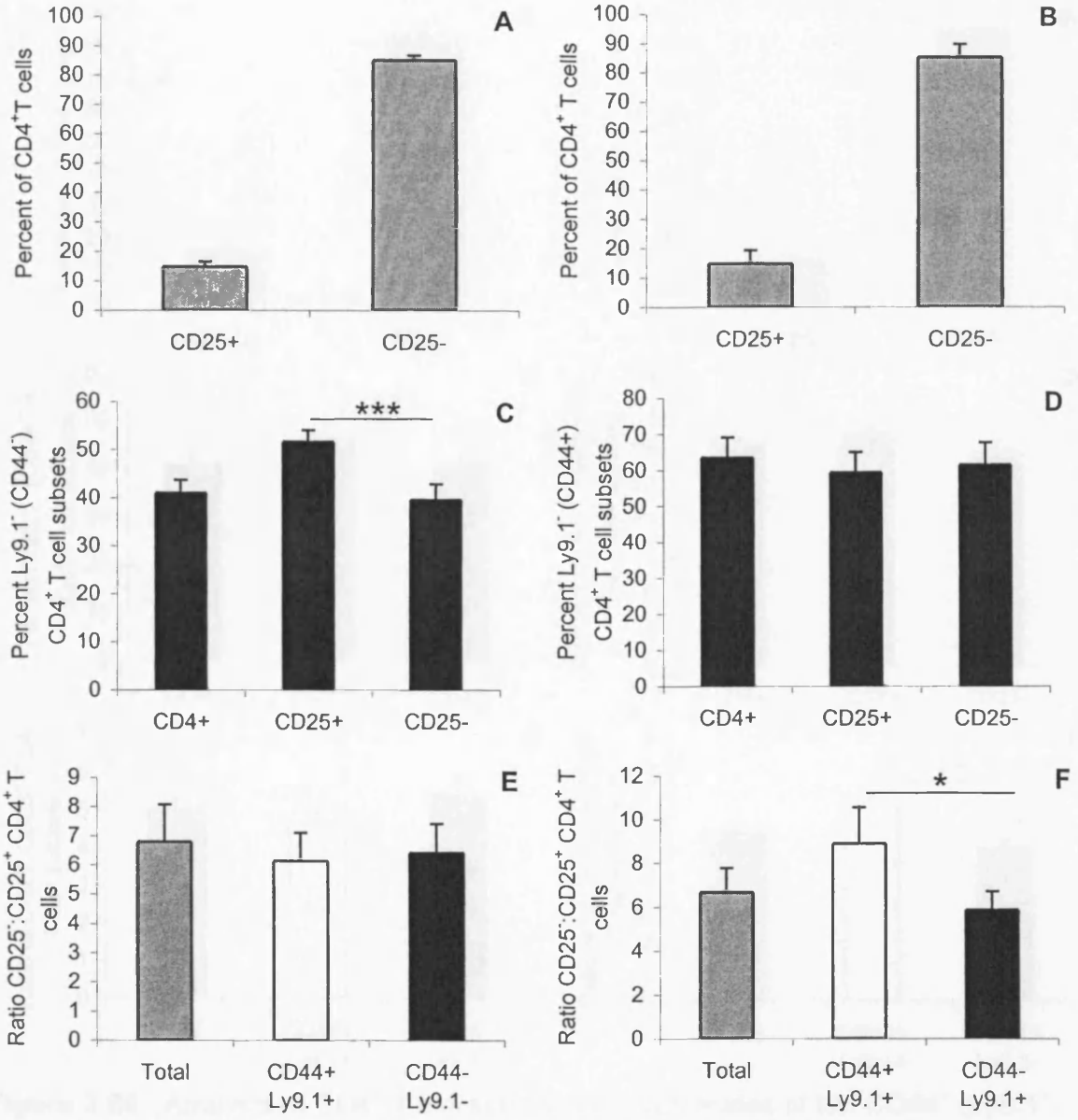

Figure 3.85. Analysis of CD4 T cell subsets in spleens of LM CD44$^+$ (Ly9.1$^+$, Ly5.1$^+$) & LM CD44$^{-/-}$ (Ly9.1$^-$, Ly5.1$^+$) / Or LM CD44$^+$ (Ly9.1$^+$, Ly5.1$^+$) & LM CD44$^+$ (Ly9.1$^-$, Ly5.1$^+$) & $\rightarrow$ CD44$^{+/+}$ (CD44$^{+/+}$, Ly9.1$^-$, Ly5.2$^+$) chimeras.

A, B. Percentages of CD25$^+$ and CD25$^-$ CD4$^+$ T cells in spleens. C, D. The percentage of Ly9.1$^-$ cells in each donor CD4$^+$ T cell subset. E, F. The CD4$^+$CD25$^-$:CD4$^+$CD25$^+$ T cell ratio among total, and donor Ly9.1$^+$ and Ly9.1$^-$ populations. A, C, E, are LM CD44$^+$ & LM CD44$^{-/-}$ $\rightarrow$ CD44$^{+/+}$ hosts, and B, D, F, are LM CD44$^+$ & LM CD44$^+$ $\rightarrow$ CD44$^{+/+}$ hosts. The data are mean $\pm$ s.e.m. obtained from 2 groups of chimeras: 1 group of 8 and 1 group of 9. P values, 2-sample t test; **, P<0.01, ****, P<0.001.

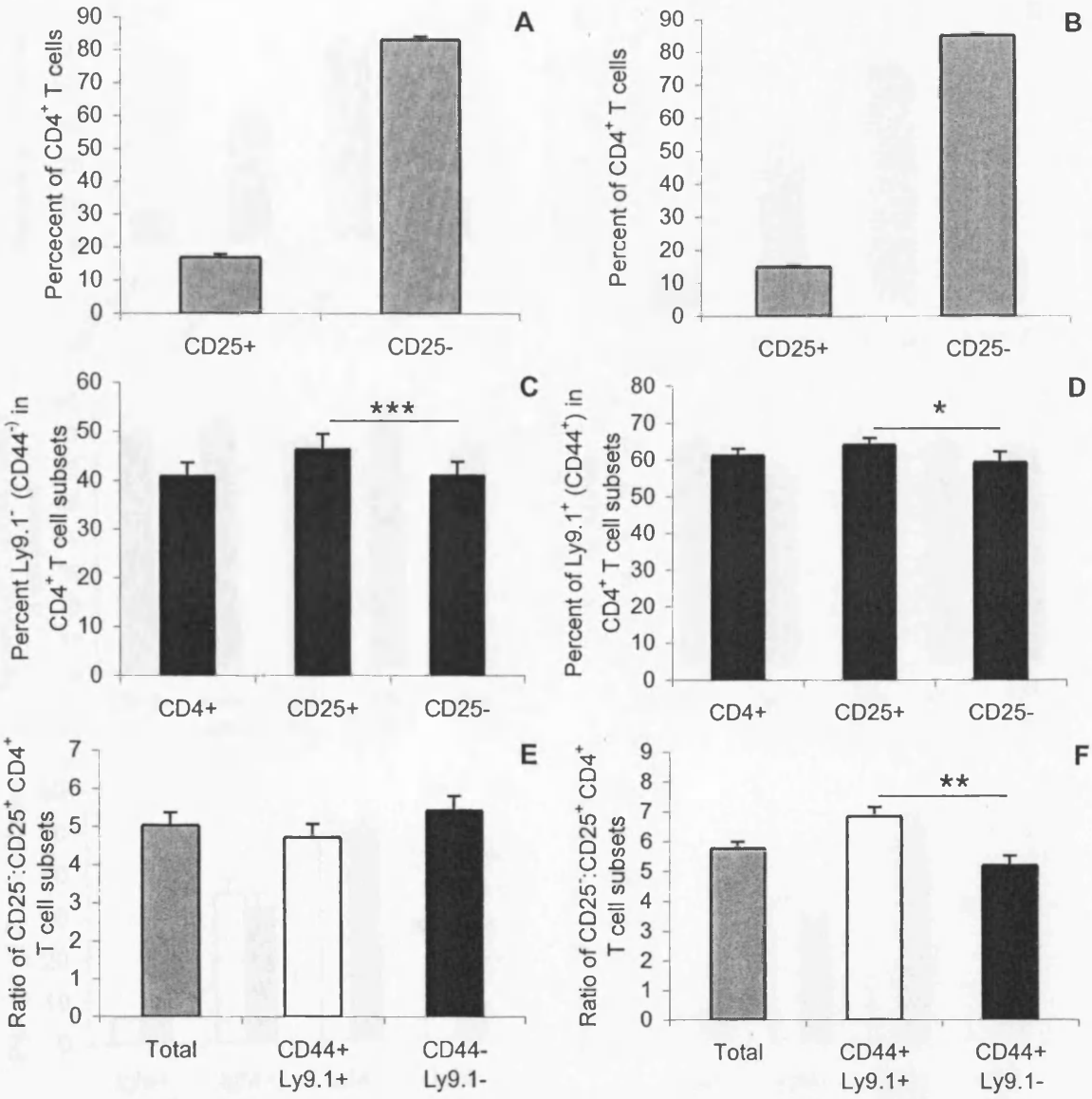

Figure 3.86. Analysis of $CD4^+$ T cell subsets in lymph nodes of LM $CD44^+$ ($Ly9.1^+$, $Ly5.1^+$) & LM $CD44^{-/-}$ ($Ly9.1^-$, $Ly5.1^+$) / Or LM $CD44^+$ ($Ly9.1^+$, $Ly5.1^+$) & LM $CD44^+$ ($Ly9.1^-$, $Ly5.1^+$) & → $CD44^{+/+}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.2^+$) chimeras.

A, B. Percentages of $CD25^+$ and $CD25^-$ $CD4^+$ T cells in lymph nodes. C, D. The percentage of $Ly9.1^-$ cells in each donor $CD4^+$ T cell population. E, F. The $CD4^+CD25^-$:$CD4^+CD25^+$ T cell ratio among $Ly9.1^+$ and $Ly9.1^-$ donor populations. A, C, E, are LM $CD44^+$ & LM $CD44^{-/-}$ → $CD44^{+/+}$ hosts, and B, D, F, are LM $CD44^+$ & LM $CD44^+$ → $CD44^{+/+}$ hosts. The data are mean ± s.e.m. obtained from 2 groups of chimeras; I group of 9 and one group of 8. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ****, $P<0.001$

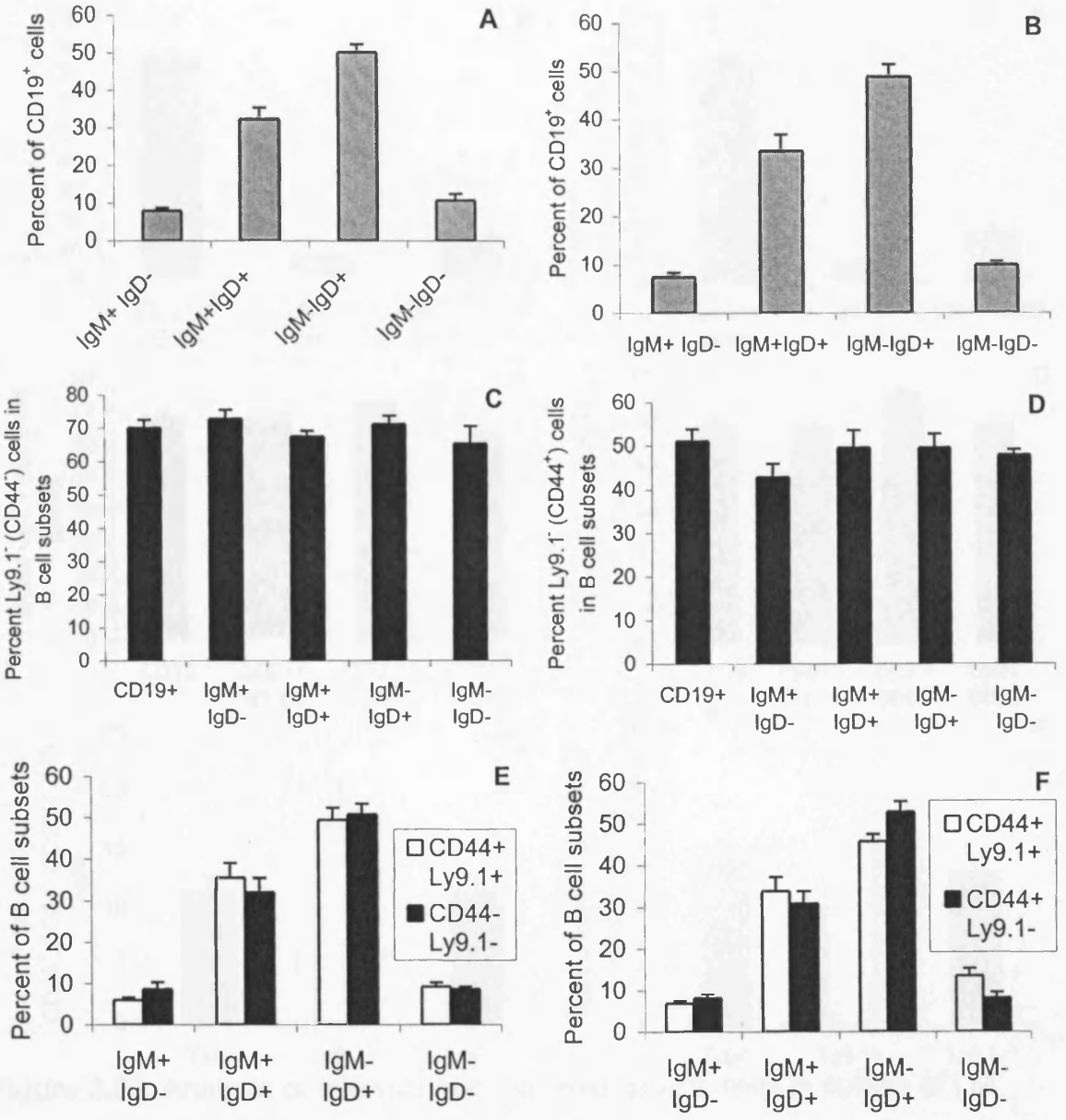

Figure 3.87. Analysis of B cell subsets in spleens of LM CD44$^+$ (Ly9.1$^+$, Ly5.1$^+$) & LM CD44$^{-/-}$ (Ly9.1$^-$, Ly5.1$^+$) / Or LM CD44$^+$ (Ly9.1$^+$, Ly5.1$^+$) & LM CD44$^+$ (Ly9.1$^-$, Ly5.1$^+$) & → CD44$^{+/+}$ (CD44$^{+/+}$, Ly9.1$^-$, Ly5.2$^+$) chimeras.

A, B. Percentages of splenic B cell (CD19$^+$) subsets defined by sIgM and sIgD expression. C, D. The percentage of Ly9.1$^-$cells in each B cell subset. E, F. Distribution of sIgM and sIgD staining among Ly9.1$^+$ or Ly9.1$^-$ CD19$^+$ cells. A, C, E, are LM CD44$^+$ & LM CD44$^{-/-}$ → CD44$^{+/+}$ hosts, and B, D, F, are LM CD44$^+$ & LM CD44$^+$ → CD44$^{+/+}$ hosts. The data are mean ± s.e.m. obtained from 2 groups of chimeras: 1 group of 8 and 1group of 9.

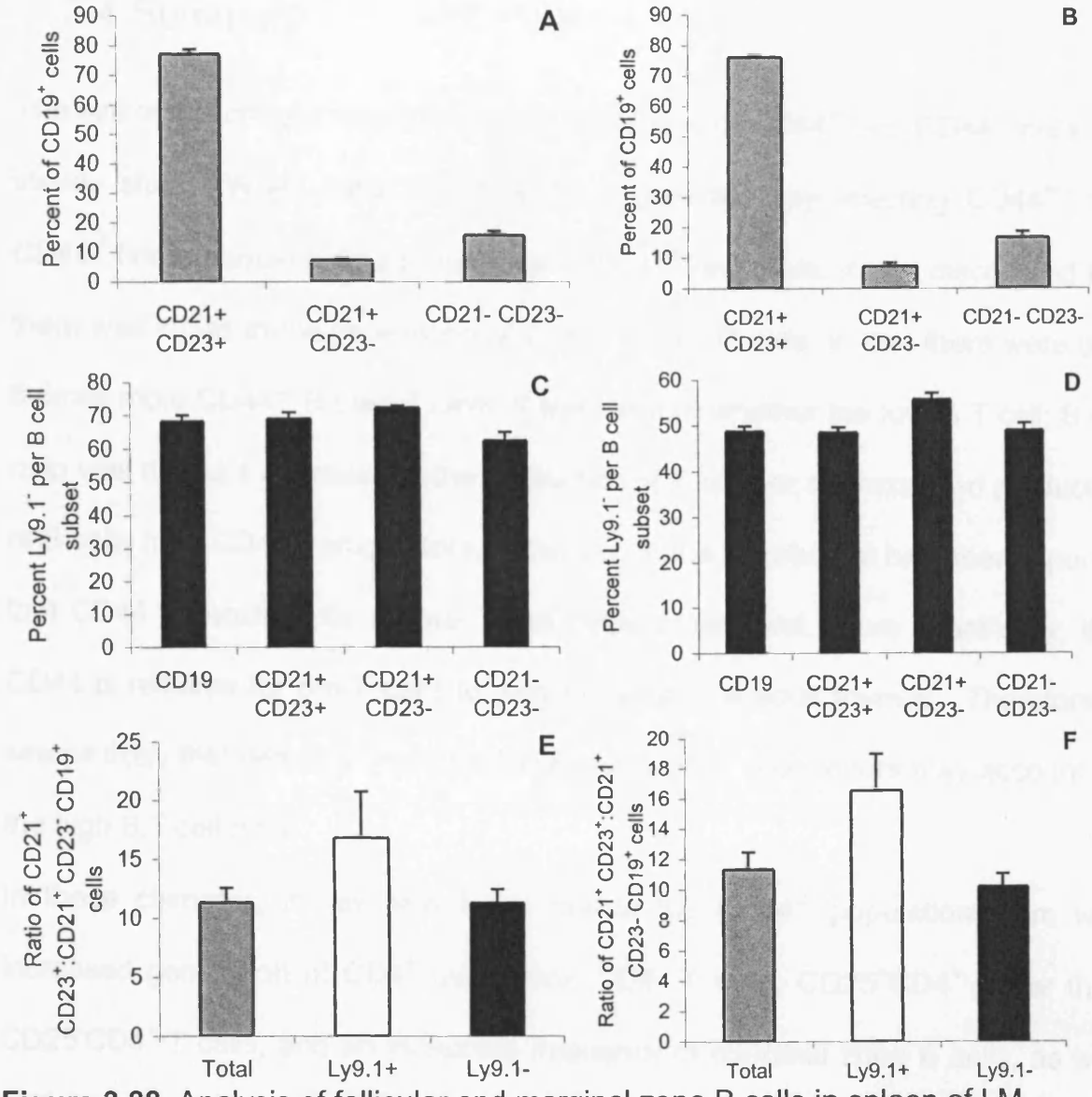

Figure 3.88. Analysis of follicular and marginal zone B cells in spleen of LM $CD44^{+}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) & LM $CD44^{-/-}$ ($Ly9.1^{-}$, $Ly5.1^{+}$) / Or LM $CD44^{+}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) & LM $CD44^{+}$ ($Ly9.1^{-}$, $Ly5.1^{+}$) & $\rightarrow$ $CD44^{+/+}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.2^{+}$) chimeras.

A, B. Percentages of splenic B cell ($CD19^{+}$) subsets defined by CD21 and CD23 expression. C, D. The percentage of $Ly9.1^{-}$ donor cells in each B cell subset. E, F. Ratio of $CD21^{+}$ $CD23^{+}$: $CD21^{+}$ $CD23^{-}$ cells among total, $Ly9.1^{-/-}$ and $Ly9.1^{+}$ $CD19^{+}$ cells. A, C, D, are LM $CD44^{+}$ & LM $CD44^{-/-}$ $\rightarrow$ $CD44^{+/+}$ hosts, and B, D, F, are LM $CD44^{+}$ & LM $CD44^{+}$ $\rightarrow$ $CD44^{+/+}$ hosts. The data are mean $\pm$ s.e.m. obtained from 2 groups of chimeras: 1 group of 8 and 1 group of 9. P values, 2-sample t test; *, $P<0.05$

3.4.Summary

The aim of this chapter was to compare the fitness of $CD44^{+/+}$ vs. $CD44^{-/-}$ cells in a steady state. When initial chimeras were generated by injecting $CD44^{+/+}$ and $CD44^{-/-}$ bone marrow cells into irradiated $CD44^{+/+}$ recipients, it was discovered that there was a bias in the generation of $CD44^{-/-}$ T and B cells, in that there were over 6 times more $CD44^{-/-}$ B than T cells. It was unclear whether the low in T cell: B cell ratio was due to a decrease in the production of T cells or an increased production of B cells from $CD44^{-/-}$ progenitors. However, in the literature, it has been reported that CD44 is required for normal T cell development and, more specifically, that CD44 is required for pre-T cells to gain entry into the adult thymus. Therefore, it seems likely that deficient T cell production by $CD44^{-/-}$ progenitors may account for the high B:T cell ratio.

In these chimeras, it was also found that in the $CD44^{-/-}$ population there was increased generation of $CD4^+$ rather than $CD8^+$ T cells, $CD25^+CD4^+$ rather than $CD25^-CD4^+$ T cells, and an increased frequency of marginal zone B cells, as well as a decreased proportion of B1-a B cells. With regard to this latter finding, it should be noted that peritoneal B cells / B-1 cells are thought to be self-renewing cells derived from the foetal liver. Hence, it was surprising to find B1 cells derived from donor bone marrow present in the mixed chimeras. Until this phenomenon could be shown to be true and not an artefact, no further studies were carried out.

When mixed bone marrow chimeras were similarly made in $CD44^{-/-}$ hosts, there was still a significant difference between the $CD44^{+/+}$ progenitors and $CD44^{-/-}$ progenitors in their ability to develop into B vs. T cells, $CD4^+$ vs. $CD8^+$ T cells, $CD25^+CD4^+$ vs. $CD25^-CD4^+$T cells, and marginal zone vs. follicular B cells. These

findings indicated that the observed biases in lymphocyte generation were not dependent on CD44 expression in the host. This was of interest, since one target for CD44 binding is thought to be hyaluronic acid bound to CD44 on the surface of other cells.

To investigate the extent to which reduced production of $CD44^{-/-}$ T cells in mixed chimeras simply reflected on an inability of $CD44^{-/-}$ progenitors to enter the thymus (as opposed to competition from $CD44^{+/+}$ progenitors), chimeras were made by injecting $CD44^{-/-}$ bone marrow alone into irradiated $CD44^{+/+}$ hosts. When this was done, only a slight deficiency in ability of the $CD44^{-/-}$ progenitors to develop into T cells in the $CD44^{+/+}$ host thymus was observed; this was demonstrated by a number amount of total splenic $CD3^{+}$ cells compared to $CD44^{+/+} \rightarrow CD44^{+/+}$ chimeras. This experiment suggested that $CD44^{-/-}$ progenitor T cells can enter the thymus and can reconstitute a wild type mouse, and that the major deficit in $CD44^{-/-}$ T cells in the mixed chimeras, was related to direct competition between $CD44^{-/-}$ and $CD44^{+/+}$ progenitors. It also became apparent that there was a significant proportion of radioresistant host T cells present after 900 rads irradiation. Since host T cells were not distinguishable from donor $CD44^{+/+}$ cells in the initial mixed chimeras, their presence could have led to an overestimate of the deficiency of $CD44^{-/-}$ T cell generation.

Radioresistance was assessed in both $CD44^{+/+}$ hosts and $CD44^{-/-}$ hosts by reconstituting irradiated (900 rad) recipients with only $CD44^{+/+}$ bone marrow, to minimise potential complications of $CD44^{-/-}$ progenitor deficiencies. There were a significant number of radioresistant T cells in the spleen and to a lesser extent in the LN in both strains. Slightly more $CD8^{+}$ than $CD4^{+}$ T cells persisted after 900rad

irradiation. Therefore, some of the apparent biased contribution of $CD44^{-/-}$ cells to the $CD4^+$ T cell population in the reconstituted mixed chimeras may have been due to the relative radioresistance of host $CD8^+$ T cells. Conversely, $CD25^+CD4^+$ T cells appeared to be slightly more radioresistant than $CD25^-CD4^+$ T cells. This latter finding indicated that the greater contribution of $CD44^{-/-}$ cells to the $CD25^+CD4^+$ population was, if anything, underestimated in the mixed chimeras. Of note, the same differential radioresistance was not found in the $CD44^{-/-}$ host chimeras, where the $CD4^+$ T cells were more radioresistant and there was no difference between the $CD25^+CD4^+$ and $CD25^-CD4^+$ populations. Overall there were less radioresistant cells found in the LN of these mice, and therefore the composition of cells present in the $CD44^{-/-}$ host chimeras were more representative of donor derived cells, with less of an influence from host radioresistant cells. Significant numbers of radioresistant B cells were not observed in any hosts given 900 rads irradiation.

Due to the presence of radioresistant T cells, another allotypic marker was required to fully distinguish donor and host cells. For this purpose, expression of Ly9.1 was investigated and found suitable; Ly9.1 was expressed on all lymphocytes being studied, and unlike CD2 was expressed at early stages of lymphocyte development.

Another approach to decreasing the possible contamination of radioresistant T cells on the results was to increase the amount of irradiation given. However, treating recipients with 1100 rads reduced but did not eliminate radioresistant T cells. Therefore, the allotypic markers Ly9.1, Ly5.1, Ly5.2 were used in combination to differentiate the three populations of T cells. The chimeras

confirmed the previous results, namely that the $CD44^{-/-}$ cells were found preferentially among B cells vs. T cells, $CD4^+$ vs. $CD8^+$ T cells, $CD25^+CD4^+$ vs. $CD25^-CD4^+$ T cells and marginal zone vs. follicular B cells; these results were obtained on focusing specifically on donor-derived cells. As a note of caution for these experiments, 1100 rads is not an ideal amount of irradiation to routinely use, for the damage to the stromal layer increases with the amount of irradiation given. As the stromal layer of the thymus and bone marrow is essential in the development and differentiation of lymphocytes, minimal necessary doses of irradiation should be given.

Subsequently, mixed chimeras were generated in $RAG^{-/-}$ hosts. As these mice do not possess mature T and B cells, a lower amount of irradiation can be used which also reduces the damage to the stromal layer. In addition, using $RAG^{-/-}$ hosts eliminates any possible influence of mature T cells on the ability of donor marrow to reconstitute lymphocyte populations. The $RAG^{-/-}$ host chimeras confirmed the previous results, showing that the $CD44^{-/-}$ progenitors had an increased tendency to generate B2 B cells, $CD4^+$ T cells, $CD25^+CD4^+$ T cells and marginal zone B cells. There was also a relative deficit in the production of $CD44^{-/-}$ B1a B cells of the peritoneal cavity and spleen as seen in the original chimeras. Since B cells present in $RAG^{-/-}$ host chimeras can only be derived from donor bone marrow this result implies that B1 B cell precursors are present in marrow as well as foetal liver. However an alternative explanation is that circulating B1 cells were present in the bone marrow preparations, these cells were capable of reconstituting the B1 population in the host due to their self-renewing capabilities.

To investigate further the nature of the competition between $CD44^{-/-}$ and $CD44^{+/+}$ progenitors, reconstitution of lymphocytes was studied using different ratios of $CD44^{+/+}$ and $CD44^{-/-}$ bone marrow cells in the reconstituting inoculum. The contribution of $CD44^{-/-}$ cells to all lymphocyte subsets increased as the fraction of these cells in the bone marrow inoculum increased, which is consistent with there being competition between progenitor cells for developmental niches. However, the biased production of lymphocyte sub-populations from $CD44^{-/-}$ precursors was observed at all $CD44^{-/-}$:$CD44^{+/+}$ starting ratios (From 1:3 to 3:1). This result implies a relatively high competitive advantage/disadvantage of CD44 expression in biasing production of these cells.

Among different chimeras, the percentage of $CD44^{-/-}$ cells present in the mature T and B cells populations has been variable, even though the trends with respect to biased production of lymphocyte sub-populations have been consistent. One variable that could contribute to the differing levels of reconstitution is the number of haematopoietic stem cells (HSC) present in different bone marrow preparations. This was controlled for by generating chimeras in $RAG^{-/-}$ hosts, by the injection of enriched HSC mixed bone marrow, that is bone marrow that has had the lineage positive cells removed. Significantly, the resulting chimeras still had all the bias found previously in the mixed chimeras (↑B cells, ↑$CD4^+$ T cells, ↑$CD25^+CD4^+$ T cells, ↑marginal zone B cells, and ↓B1a B cells). Interestingly, despite injecting putative HSC at a 1:1 ratio of $CD44^{-/-}$ and $CD44^{+/+}$ cells, the overall reconstitution with $CD44^{-/-}$ cells was relatively low (e.g. 35% of B cells were $CD44^{-/-}$). This could mean that $CD44^{-/-}$ HSC are relatively poor at reconstituting lymphocytes, or that

more contaminating non-HSC are found in this phenotypic population in $CD44^{-/-}$ bone marrow.

To control for the possible differences in background genes between $CD44^{-/-}$ and $CD44^{+/+}$ mice that could contribute to the biased production of T and B cells observed in mixed chimeras, additional chimeras were made using F2 offspring of matings between $CD44^{+/+}$ (C57Bl/6) and $CD44^{-/-}$ mice (littermates, LM) as a source of bone marrow; this was necessary because the $CD44^{-/-}$ mice were not fully backcrossed onto a C57Bl/6 background and still have some 129Sv characteristics, such as $Ly9.1^{+}$ expression remaining. Mixed chimeras generated using $CD44^{+}$ and $CD44^{-/-}$ LM bone marrow yielded, for the most part, results that were consistent with those obtained in other chimeras. Thus, in general $CD44^{-/-}$ cells were biased in the production of B cells vs. T cells, $CD4^{+}$ vs. $CD8^{+}$ T cells, $CD25^{+}CD4^{+}$ vs. $CD25^{-}CD4^{+}$ T cells and marginal zone vs. follicular B cells (note that B1a B cells were not examined in these chimeras). However, these results were confounded by the observation that balanced production of T and B cell subsets did not occur in the control mixed LM chimeras. In these mice, generated by injecting $Ly9.1^{+}CD44^{+}$ and $Ly9.1^{-}CD44^{+}$ bone marrow into irradiated $CD44^{+/+}$ ($Ly5.2^{+}$) mice, uneven reconstitution of subsets was seen, although this did not resemble the pattern observed in $CD44^{-/-}$ and $CD44^{+} \rightarrow CD44^{+/+}$ mixed LM chimeras. For example, $Ly9.1^{+}$ cells appeared to preferentially generate B cells over T cells and follicular B cells over marginal zone B cells, while showing no bias in $CD4^{+}$ vs. $CD8^{+}$ T cells and a bias with regard to $CD25^{+}CD4^{+}$ T cells, depending on whether spleen or LN were examined.

The results in these control chimeras could indicate that either multiple genes are controlling the biased production of the different lymphocyte subsets, and that these segregate differentially in the F2 offspring. However if it were the case, one would have expected a more variable result in the $CD44^{-/-}$ and $CD44^{+}$ mixed littermate chimeras.

Therefore in conclusion, while the variability in the control littermate chimeras is of potential concern, the overall consistency in the results generated in a variety of mixed chimeras supports the idea that CD44 plays an important role in biasing lymphocyte development.

4. Chapter 4: Homing and altered adhesion profiles of $CD44^{-/-}$ lymphocytes when placed in direct competition with $CD44^{+/+}$ lymphocytes.

4.1. Introduction.

The main function for CD44 is as a cellular homing and adhesion receptor involved in lymphocyte migration and stem cell homing. As well as hyaluronate CD44 can also bind other extracellular matrix components, such as chondriton sulphate, heparin sulphate, fibrinogen, serglycin, and osteopontin; binding these ligands can also facilitate cell motility and adhesion. CD44 is thought to be involved in the homing and migration of lymphocytes by tethering the cells to the vessel wall via hyaluronate, and/or other extracellular matrix proteins. This adhesion supports the initiation of lymphocyte rolling and allows the selectin and integrin attachments to proceed (229-233). The attachment of lymphocytes to the endothelial walls allows the extravasation into the target organs and inflamed tissues. This process is facilitated by the cytoplasmic domain of CD44, which is present in lipid rafts on the cell surface lead to changes in cellular morphology via the interaction of CD44 via the cytoskeleton thus aiding extravasation. (123, 162, 195, 234-236). A more comprehensive review of the function of CD44 and its ligands can be found in Section 1.3.

CD44 has also been shown to have a role in stem cell homing and in the seeding of progenitors to the bone marrow and thymus (221, 224, 225, 237, 238) This has also been described previously in section 1.3 and briefly in Section 3.1.

In the previous chapter it was observed that when $CD44^{-/-}$ progenitors were placed in direct competition with $CD44^{+/+}$ progenitors, a bias in the production of T and B cells and some of their subsets was apparent. The $CD44^{-/-}$ cells were found preferentially amongst B cells vs. T cells, $CD4^+$ vs. $CD8^+$ T cells, $CD25^+CD4^+$ vs. $CD25^-CD4^+$ T cells and marginal zone vs. follicular B cell populations, there was also a deficit in the B1a B cells population of $CD44^{-/-}$ cells. The unequal production of $CD44^{-/-}$ and $CD44^{+/+}$ populations was found in numerous chimeras, when using $CD44^{+/+}$ and $CD44^{-/-}$ progenitors at different ratios (Sections 3.3.1, 3.3.3 & 3.3.8), and the biases observed were independent of the host environment, as they were observed when either $CD44^{-/-}$ or $CD44^{+/+}$ mice were used as hosts (Section 3.3.2). Experimental evidence has shown that $CD44^{+/+}$ is necessary for homing to both the bone marrow and thymus. It has also been demonstrated that $CD44^{hi}$ cells home better to the bone marrow than $CD44^{lo}$ cells when in competition for bone marrow seeding (239). Although this was demonstrated using $CD8^+$ T cells, this and previous reports raises the possibility that there may be a deficiency in the capability of the $CD44^{-/-}$ bone marrow to seed the thymus and/or when inoculated in conjunction with $CD44^{+/+}$ bone marrow. *In vitro* experiments have demonstrated that HSC bind rapidly to the bone marrow stroma (240) As the extracellular matrix within the bone marrow has a high proportion of the CD44 ligands hyaluronate and fibrinogen, which have been secreted by the stromal cell layer, $CD44^{-/-}$ and $CD44^{+/+}$ progenitors might differ in their efficiency to seed the bone marrow. CD44 is not the only protein that can bind hyaluronate. For example, RHAMM also binds hyaluronate. However, it does so with five times less affinity then CD44, would make such 'backup' strategies much less efficient. Moreover, in one study it was

demonstrated that leukocytes from $CD44^{-/-}$ mice could not bind to immobilised hyaluronate (241).

In adult mice, $CD44^{-/-}$ lymphocytes traffic normally under non-inflammatory conditions, and have a normal distribution within lymphoid organs (241). This fits with previous data showing that CD44 is not required for lymphocyte entry to splenic white pulp (242). As reported in the previous chapter, we found $CD44^{+/+}$ lymphocytes present in all lymphoid organs examined. In contrast CD44 may play a role in the trafficking of Langerhans cells. In $CD44^{-/-}$ mice, the cells were shown to emigrate from the epidermis but failed to reach the lymph nodes in response to an inflammatory stimulus (243).

4.2.Aim.

The aim of this chapter is to investigate two possibilities that could account for the unequal proportion of $CD44^{-/-}$ and $CD44^{+/+}$ cells observed among lymphocyte populations. We will determine: (1) whether there is a biased lymphocyte production linked to a poor ability of $CD44^{-/-}$ progenitors to efficiently enter and seed either the bone marrow or thymus, (2) whether the $CD44^{-/-}$ lymphocyte have altered homing abilities compared to those of $CD44^{+/+}$ lymphocytes when placed in competition.

4.3.Results

4.3.1. Homing of BM cells to the bone marrow

Although previous studies have suggested a requirement for CD44 in the homing of cells to the bone marrow, the results in the previous chapter did not indicate any deficiency in the ability of the $CD44^{-/-}$ cells to generate B cells in mixed bone marrow chimeras. On the other hand, reduced homing of progenitors to the thymus might have contributed to the lower production of $CD44^{-/-}$ T cells. To compare directly the ability of the $CD44^{+/+}$ and $CD44^{-/-}$ progenitor cells to home to the bone marrow and thymus, lineage depleted bone marrow cells were obtained from $CD44^{+/+}$ and $CD44^{-/-}$ mice using a cocktail of antibodies against cell lineage specific molecules and dynal beads to remove all cells bound to antibody. $CD44^{+/+}$ cells or $CD44^{-/-}$ cells were CFSE labelled and mixed with unlabelled $CD44^{-/-}$ or $CD44^{+/+}$ cells respectively and inoculated into irradiated (450 rad) $CD44^{+/+}$ hosts. The same number of labelled $CD44^{+/+}$ or $CD44^{-/-}$ bone marrow cells was also injected alone into $CD44^{+/+}$ recipients to analyse homing in the absence of competition from the other cell type.

Mice were sacrificed 12, 24, 48 hours after inoculation, and their bone marrow and thymus removed. Blood lymphocytes were also collected at 12 hours post inoculation. A sample of cells was analysed by FACS to measure the amount of CFSE labelled cells present in the tissue.

CFSE labelled cells were not detected in the thymus at any time points, but were found in the bone marrow of all mice. There was no significant difference in the percentage of $CD44^{+/+}$ and $CD44^{-/-}$ bone marrow cells recovered, whether they were injected individually or in conjunction with each other (Figure 4.1A).

There was a difference however, in the percentages of CFSE positive cells isolated from the blood of the mice sacrificed 12hr-post inoculation (Figure 4.1B). There were significantly more $CD44^{+/+}$ cells in the blood than $CD44^{-/-}$ cells when injected individually, but there were significantly more $CD44^{-/-}$ than $CD44^{+/+}$ cells in the blood when injected in conjunction with each other. Predictably there were more $CD44^{+/+}$ cells recovered when injected alone than with $CD44^{-/-}$ cells, as there were twice as many $CD44^{+/+}$ cells injected initially. However, this was not observed with the $CD44^{-/-}$ cells, where more $CD44^{-/-}$ cells were found in the blood of mice co-injected with unlabelled $CD44^{+/+}$ cells than those inoculated solely with $CD44^{-/-}$ cells.

CFSE also has the advantage that the MFI decreases with cell division. Therefore, it is possible to evaluate whether the cells injected have divided in the mouse (Figure 4.1C). It was only possible to compare $CD44^{+/+}$ and $CD44^{-/-}$ cells individually, due to a difference in the initial CFSE staining intensity. There was significant lower CFSE intensity in both the $CD44^{+/+}$ and $CD44^{-/-}$ cells when injected alone compared to that when cells were mixed with unlabelled cells of the recipient phenotype, at both 12 and 24 hours post injection. This difference was not observed 48hrs post-injection.

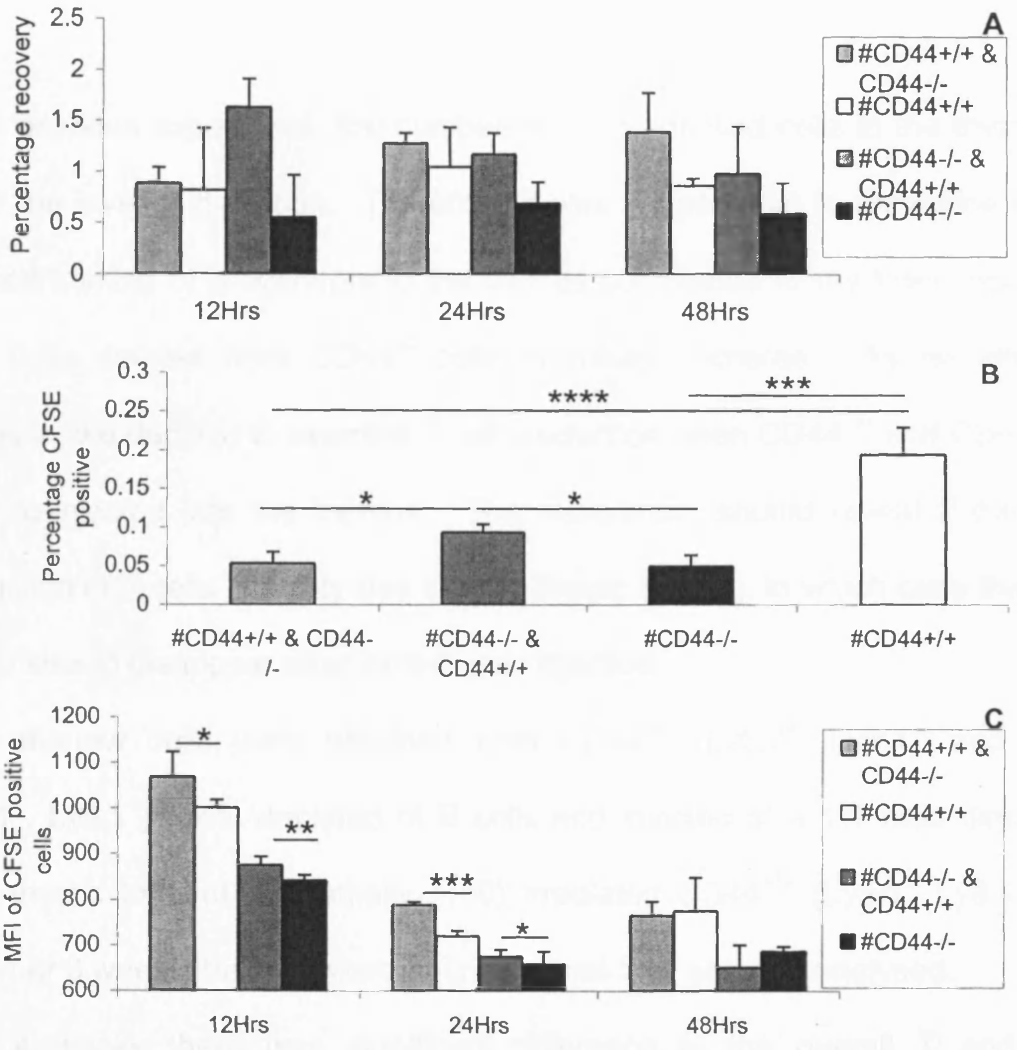

Figure 4.1. Analysis of $CD44^{+/+}$ and $CD44^{-/-}$ progenitor homing after i.v. injection.

A. Recovery of CFSE labelled cells in the bone marrow expressed as a percentage of cells initially injected. B. Percentage of CFSE positive cells found in the blood 12h-post injection. C. MFI of CFSE intensity of recovered bone marrow cells at various time points after inoculation. The data are mean ± s.e.m from groups of 3. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$. # indicates CFSE labelled cells. MFI, mean fluorescence intensity.

4.3.2. Intra-thymic injections

In the previous experiment, the number of CFSE-labelled cells in the thymus was below the level of detection. Therefore, it was not possible to determine whether reduced homing of progenitors to the thymus contributed to the lower percentage of T cells derived from $CD44^{-/-}$ cells in mixed chimeras. As an alternative approach, we decided to examine T cell production when $CD44^{+/+}$ and $CD44^{-/-}$ cells were co-injected into the thymus. This experiment should reveal if the biased production of T cells is solely due to prior thymic homing, in which case the bias in T cells should disappear after intra-thymic injection.

Bone marrow cells were obtained from $CD44^{+/+}$ ($Ly5.2^{+}$, $Ly9.1^{-}$) and $CD44^{-/-}$ ($Ly5.1^{+}$, $Ly9.1^{+}$) mice, depleted of B cells and injected at a 1:1 ratio directly into each thymic lobe of sub-lethally (450) irradiated $CD44^{+/+}$ ($Ly5.1^{+}$ $Ly9.1^{-}$) mice. After 4 or 6 weeks the mice were sacrificed and their spleens analysed.

After 4 weeks there was significant difference in the overall T and B cell percentages. The mice injected with $CD44^{+/+}$ bone marrow alone had a high percentage of B cells but few T cells. There were significantly fewer B cells in the mice co-injected with $CD44^{+/+}$ and $CD44^{-/-}$ cells, and in turn statistically fewer B cells in the mice injected with $CD44^{-/-}$ cells than both the $CD44^{+/+}$ and $CD44^{-/-}$ co-injected mice or the $CD44^{+/+}$ mice. The percentage of T cells remained constant independent of reconstitution (Figure 4.2A).

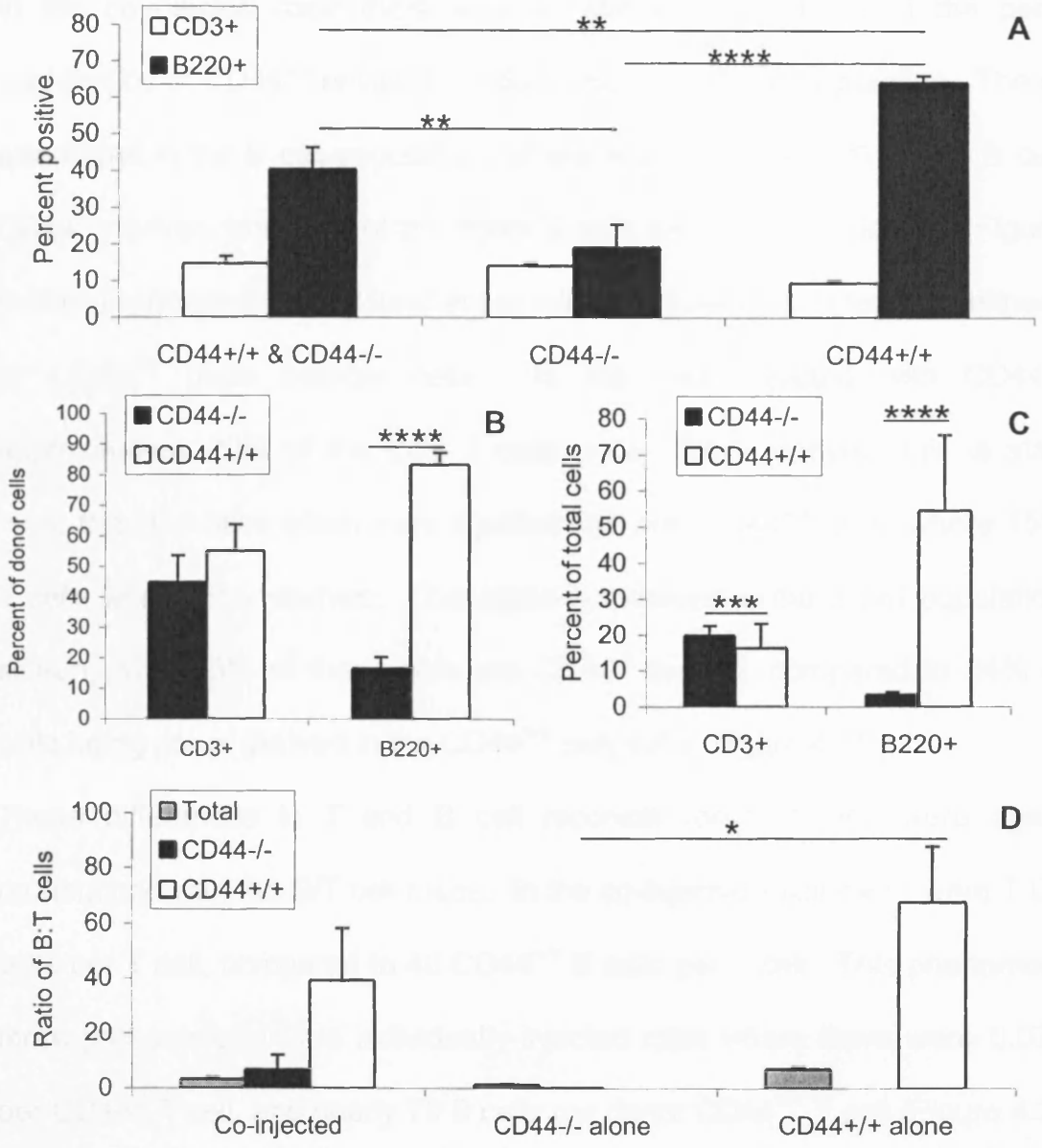

Figure 4.2. Analysis of T and B cells 4 weeks after intrathymic injection of progenitor cells

A. Percent of total T and B cells in spleen. B. Percent of donor CD44+/+ and donor $CD44^{-/-}$ cells in each splenic population from co-injection. C. Percent of donor $CD44^{+/+}$ and donor $CD44^{-/-}$ cells in each splenic population from mice injected with either $CD44^{+/+}$ or $CD44^{-/-}$ cells. D. The B:T cell ratio among total and $CD44^{+/+}$ and $CD44^{-/-}$ donor populations. The data are mean ± s.e.m from groups of 5. P values, 2-sample t test; *, $P<0.05$, ***, $P<0.005$, ****, $P<0.001$.

In the co-injected mice there was no statistical difference in the percentage contribution of $CD44^{+/+}$ or $CD44^{-/-}$ cells to the donor T cell population. The opposite was found in the B cell population, where approximately 15% of the B cells were $CD44^{-/-}$ derived, and 85% of the donor B cells were $CD44^{+/+}$ derived (Figure 4.2B). A similar phenotype was found in the mice which were injected with either $CD44^{-/-}$ or $CD44^{+/+}$ bone marrow cells. In the mice injected with $CD44^{-/-}$ cells, approximately 20% of the total T cells were $CD44^{-/-}$ derived, this is statistically more than the mice which were injected with only $CD44^{+/+}$ cells, where 15% of the T cells were donor derived. This again is reversed in the B cell population of the spleen, where 3% of the B cells are $CD44^{-/-}$ derived, compared to 54% of the B cells being donor derived in the $CD44^{+/+}$ only mice (Figure 4.2C).

These differences in T and B cell reconstitutional abilities were again more pronounced with the B/T cell ratios. In the co-injected mice there were 7 $CD44^{-/-}$ B cells per T cell, compared to 40 $CD44^{+/+}$ B cells per T cell. This phenomenon was more pronounced in the individually injected mice where there were 0.07 B cells per $CD44^{-/-}$ T cell, and nearly 70 B cells per donor $CD44^{+/+}$ T cell (Figure 4.2D).

After 6 weeks, there was little variation between the total T and B cell populations in the various groups of mice (Figure 4.3A). However, the phenotype in the co-injected mice was very different from that observed at 4 weeks, and more similar to that of the original chimeras. Approximately 20% of the donor T cells were $CD44^{-/-}$ derived and 36% of the B cells were $CD44^{-/-}$ derived (Figure 4.3B).

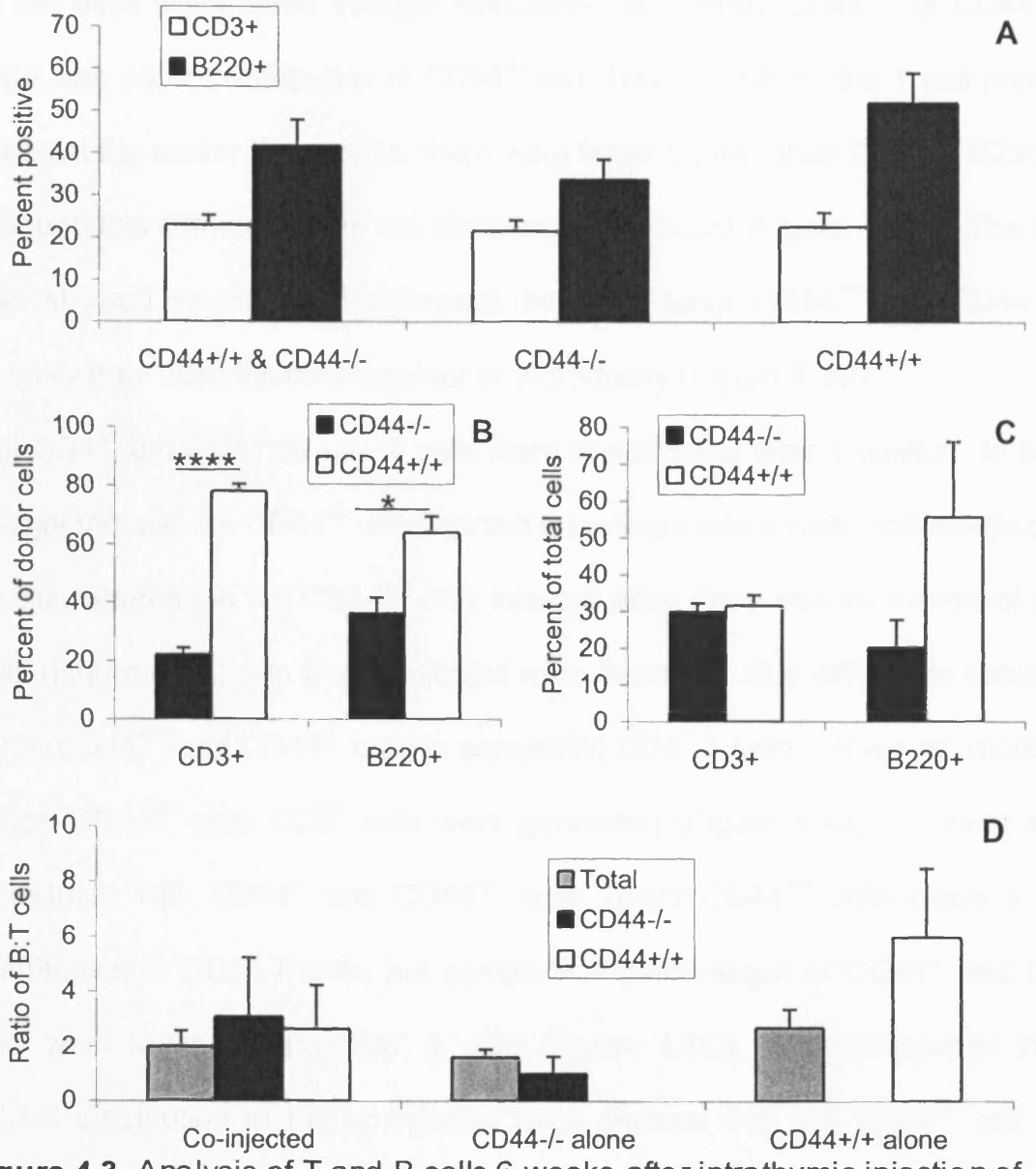

Figure 4.3. Analysis of T and B cells 6 weeks after intrathymic injection of progenitor cells

A. Percent of total T and B cells in spleen. B. Percent of donor $CD44^{+/+}$ and donor $CD44^{-/-}$ cells in each splenic population from co-injection. C. Percent of donor $CD44^{+/+}$ and donor $CD44^{-/-}$ cells in each splenic population from mice injected, with either $CD44^{+/+}$ or $CD44^{-/-}$ cells. D. The B:T cell ratio among total, and $CD44^{+/+}$ and $CD44^{-/-}$ donor populations. The data are mean $\pm$ s.e.m from groups of 5. P values, 2-sample t test; *, $P<0.05$.

In the mice which were injected individually with either $CD44^{+/+}$ or $CD44^{-/-}$ cells, there was equal contribution of $CD44^{-/-}$ and $CD44^{+/+}$ cells to the T cell population, but as at the earlier time points, there were fewer $CD44^{-/-}$ than $CD44^{+/+}$ $B220^{+}$ cells, although this difference was not statistically significant (Figure 4.3C). The B:T cell ratio showed no statistical difference between donor $CD44^{+/+}$ and $CD44^{-/-}$ cells, whether they were injected together or individually (Figure 4.3D).

The $CD4^{+}$ and $CD8^{+}$ splenic T cells were investigated after 4 weeks. In both the co-injected and the $CD44^{-/-}$ only injected mice there was a high percentage of $CD8^{+}$ T cells, whereas in the $CD44^{+/+}$ only injected mice there was an excess of $CD4^{+}$ T cells (Figure 4.4A). In the co-injected mice there was little difference between the donor $CD44^{+/+}$ and $CD44^{-/-}$ cells in generating $CD4^{+}$ T cells. However, more $CD44^{-/-}$ than $CD44^{+/+}$ cells $CD8^{+}$ cells were generated (Figure 4.4B). In mice injected individually with $CD44^{-/-}$ and $CD44^{+/+}$ cells, donor $CD44^{+/+}$ cells made a greater contribution to $CD4^{+}$ T cells, but comparable percentages of $CD44^{-/-}$ and $CD44^{+/+}$ cells were found among $CD8^{+}$ T cells (Figure 4.4C). The differences in T cell subset distribution in the co-injected mice showed that the $CD44^{-/-}$ cell ratio is significantly higher than the donor $CD44^{+/+}CD4^{+};CD8^{+}$ T cell ratio, there was no significant difference between the individually injected mice, or between the co-injected and individually injected mice (Figure 4.4D).

Among $CD4^{+}$ T cells there was no difference in the total percent of $CD25^{+}CD4^{+}$ T cells and $CD25^{-}CD4^{+}$ T cells between the groups of mice (Figure 4.6A).

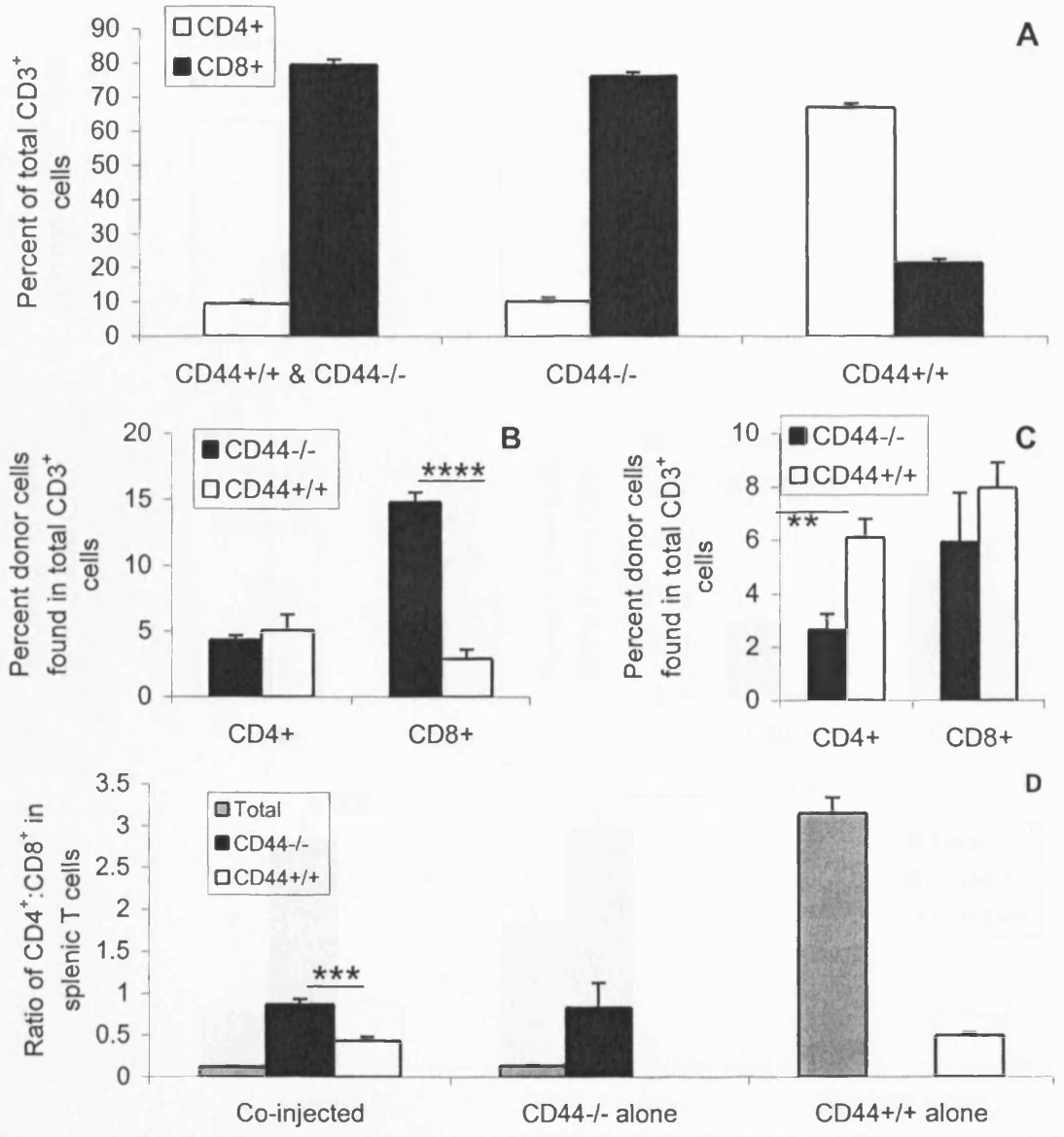

Figure 4.4. Analysis of $CD4^+$ and $CD8^+$ T cells 4 weeks after intrathymic injection of progenitor cells

A. Percent of total $CD4^+$ and $CD8^+$ T cells in the spleen. B. Percent of donor $CD44^{+/+}$ and donor $CD44^{-/-}$ cells in each splenic T cell population from mice co-injected with $CD44^{+/+}$ and $CD44^{-/-}$ cells. C. Percent of donor $CD44^{+/+}$ and donor $CD44^{-/-}$ cells in each splenic T cells subset from mice injected with either $CD44^{+/+}$ or $CD44^{-/-}$ cells. D. The $CD4^+$:$CD8^+$ T cell ratio among total, and $CD44^{+/+}$ and $CD44^{-/-}$ donor populations. The data are mean ± s.e.m from groups of 5. P values, 2-sample t test; **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$.

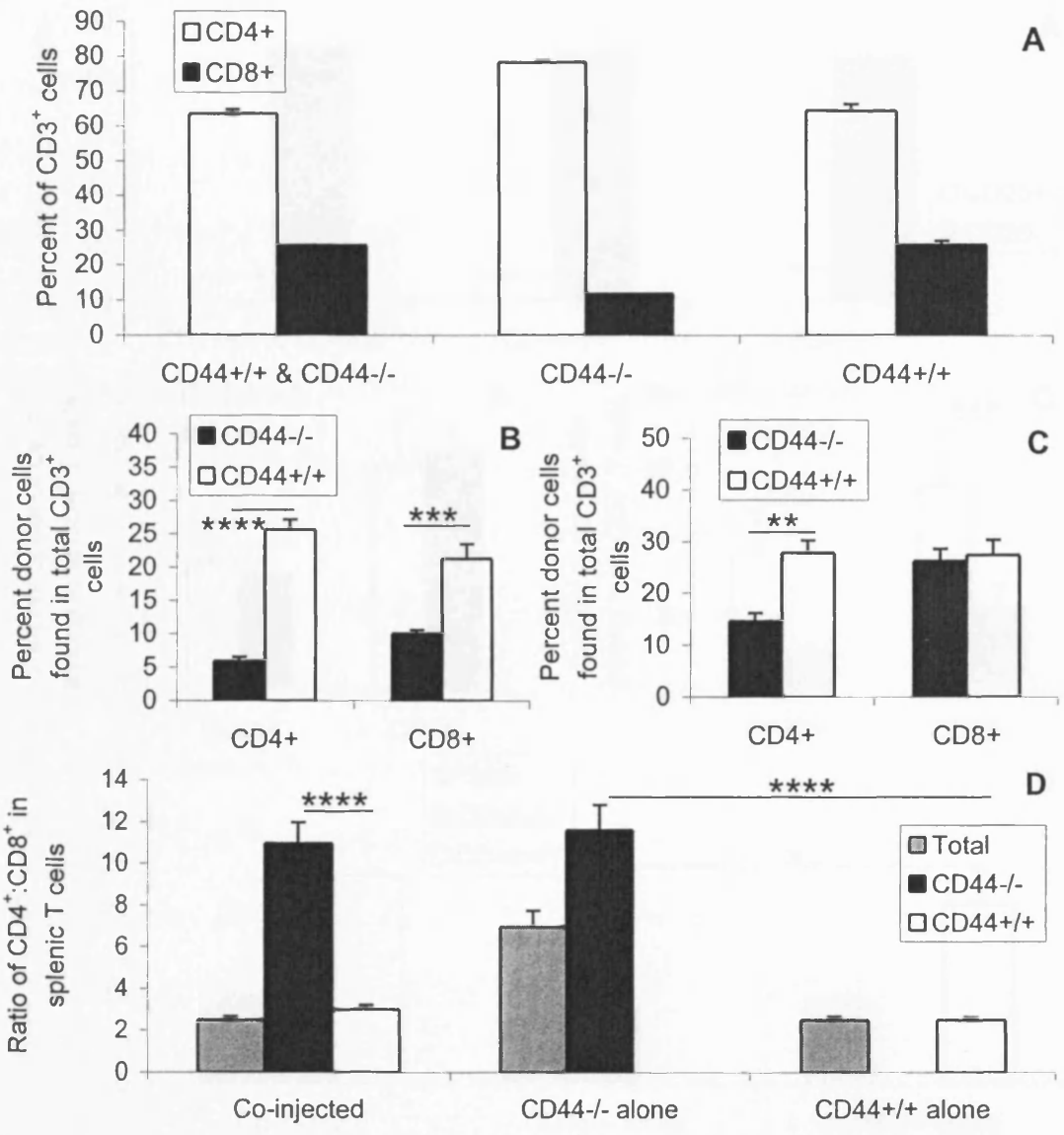

Figure 4.5. Analysis of $CD4^+$ and $CD8^+$ T cells 6 weeks after intrathymic injection of progenitor cells

A. Percent of total $CD4^+$ and $CD8^+$ T cells in the spleen. B. Percent of donor $CD44^{+/+}$ and donor $CD44^{-/-}$ cells in each splenic T cell population from mice co-injected with $CD44^{+/+}$ and $CD44^{-/-}$ cells. C. Percent of donor $CD44^{+/+}$ and donor $CD44^{-/-}$ cells in each splenic T cell population from mice injected with either $CD44^{+/+}$ or $CD44^{-/-}$ cells. D. The $CD4^+$:$CD8^+$ T cell ratio among total, and $CD44^{+/+}$ and $CD44^{-/-}$ donor populations. The data are mean ± s.e.m from groups of 5. P values, 2-sample t test; **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$.

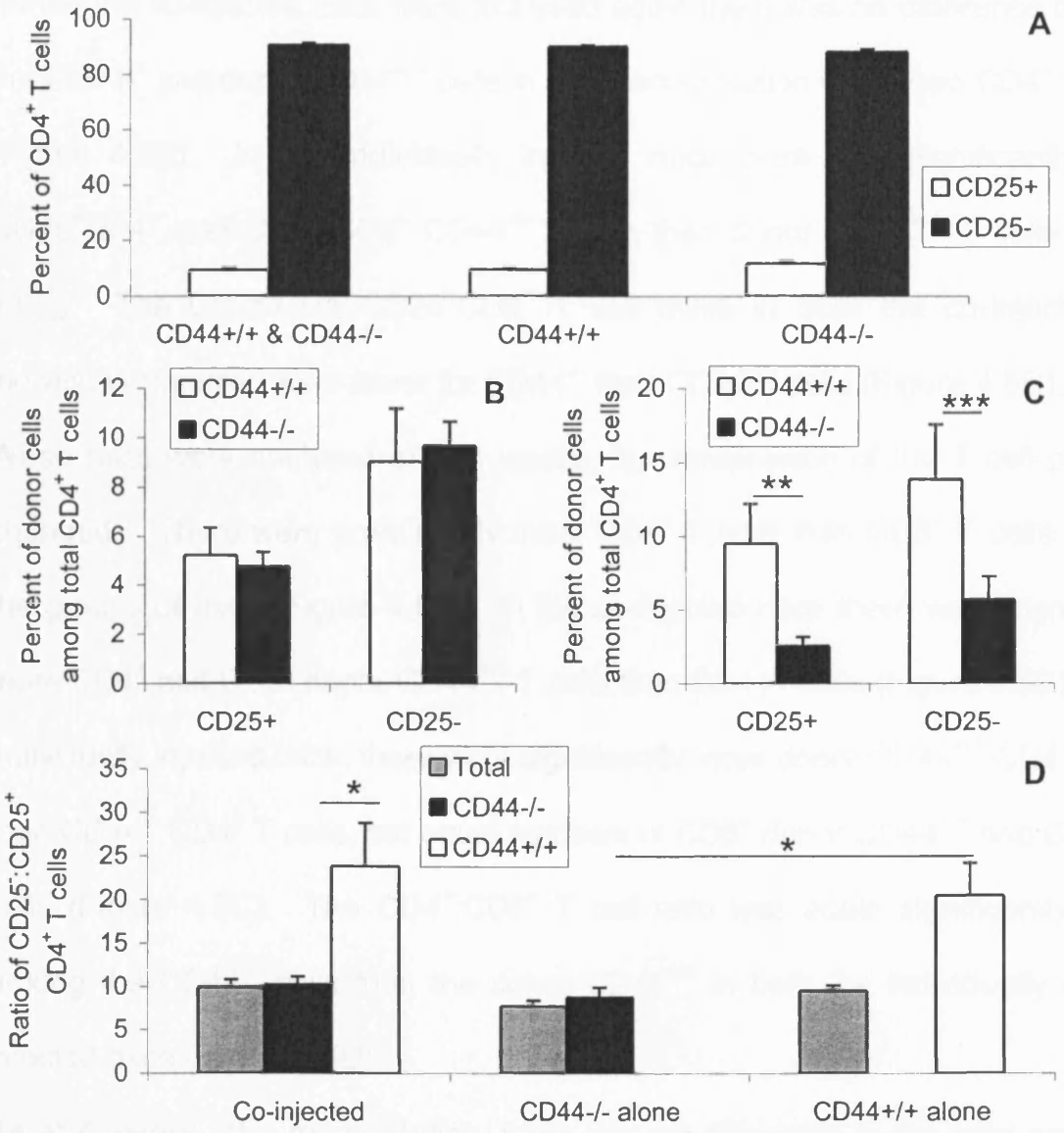

Figure 4.6. Analysis of $CD25^+$ and $CD25^-$ $CD4^+$ T cells 4 weeks after intrathymic injection of progenitor cells

A. Percent of total $CD25^+CD4^+$ and $CD25^-CD4^+$ T cells in the spleen. B. Percent of donor $CD44^{+/+}$ and donor $CD44^{-/-}$ cells in each splenic $CD4^+$ T cell subset from mice co-injected with both $CD44^{+/+}$ and $CD44^{-/-}$ cells. C. Percent of donor $CD44^{+/+}$ and donor $CD44^{-/-}$ cells in each splenic $CD4^+$ T cell subset from mice injected with either $CD44^{+/+}$ or $CD44^{-/-}$ cells. D. The $CD25^-CD4^+$:$CD25^+CD4^+$ T cell ratio among total, and $CD44^{+/+}$ and $CD44^{-/-}$ donor populations. The data are mean ± s.e.m from groups of 5. P values, 2-sample t test; *, $P<0.05$, ***, $P<0.005$.

When the co-injected mice were analysed again there was no difference between the $CD44^{-/-}$ and donor $CD44^{+/+}$ cells in their reconstitution of the two $CD4^+$ subsets (Figure 4.6B). In the individually injected mice there was significantly fewer $CD25^+CD4^+$ and $CD25^-CD4^+$ $CD44^{-/-}$ T cells than donor $CD44^{+/+}$ T cells (Figure 4.6C). The $CD25^-CD4^+$:$CD25^+CD4^+$ T cell ratios in both the co-injected and individually injected were lower for $CD44^{-/-}$ than $CD44^{+/+}$ cells (Figure 4.6D).

When mice were analysed after 6 weeks, the composition of the T cell pool had changed. There were prominently more $CD4^+$ T cells than $CD8^+$ T cells in all of the groups of mice (Figure 4.5A). In the co-injected mice there were significantly more $CD4^+$ and $CD8^+$ donor $CD44^{+/+}$ T cells than $CD44^{-/-}$ cells (Figure 4.5B). In the individually injected mice, there were significantly more donor $CD44^{+/+}$ $CD4^+$ T cells than $CD44^{-/-}$ $CD4^+$ T cells, but equal numbers of $CD8^+$ donor $CD44^{+/+}$ and $CD44^{-/-}$ T cells (Figure 4.5C). The $CD4^+$:$CD8^+$ T cell ratio was again significantly higher among the $CD44^{-/-}$ cells than the donor $CD44^{+/+}$ in both the individually and co-injected mice (Figure 4.5D).

As at 4 weeks after reconstitution, there was no difference in the total percent of $CD25^+CD4^+$ T cells and $CD25^-CD4^+$ T cells between the groups of mice (Figure 4.7A). In the co-injected mice, there were significantly fewer $CD25^+CD4^+$ and $CD25^-CD4^+$ $CD44^{-/-}$ T cells than donor $CD44^{+/+}$ T cells (Figure 4.7B). This was also the case in mice injected individually with $CD44^{-/-}$ vs. $CD44^{+/+}$ cells, although differences were not statistically significant (Figure 4.7C). The $CD25^-CD4^+$:$CD25^+CD4^+$ T cell ratios, showed little difference between the $CD44^{-/-}$ and donor $CD44^{+/+}$ cells, whether they were injected individually or together (Figure 4.7D).

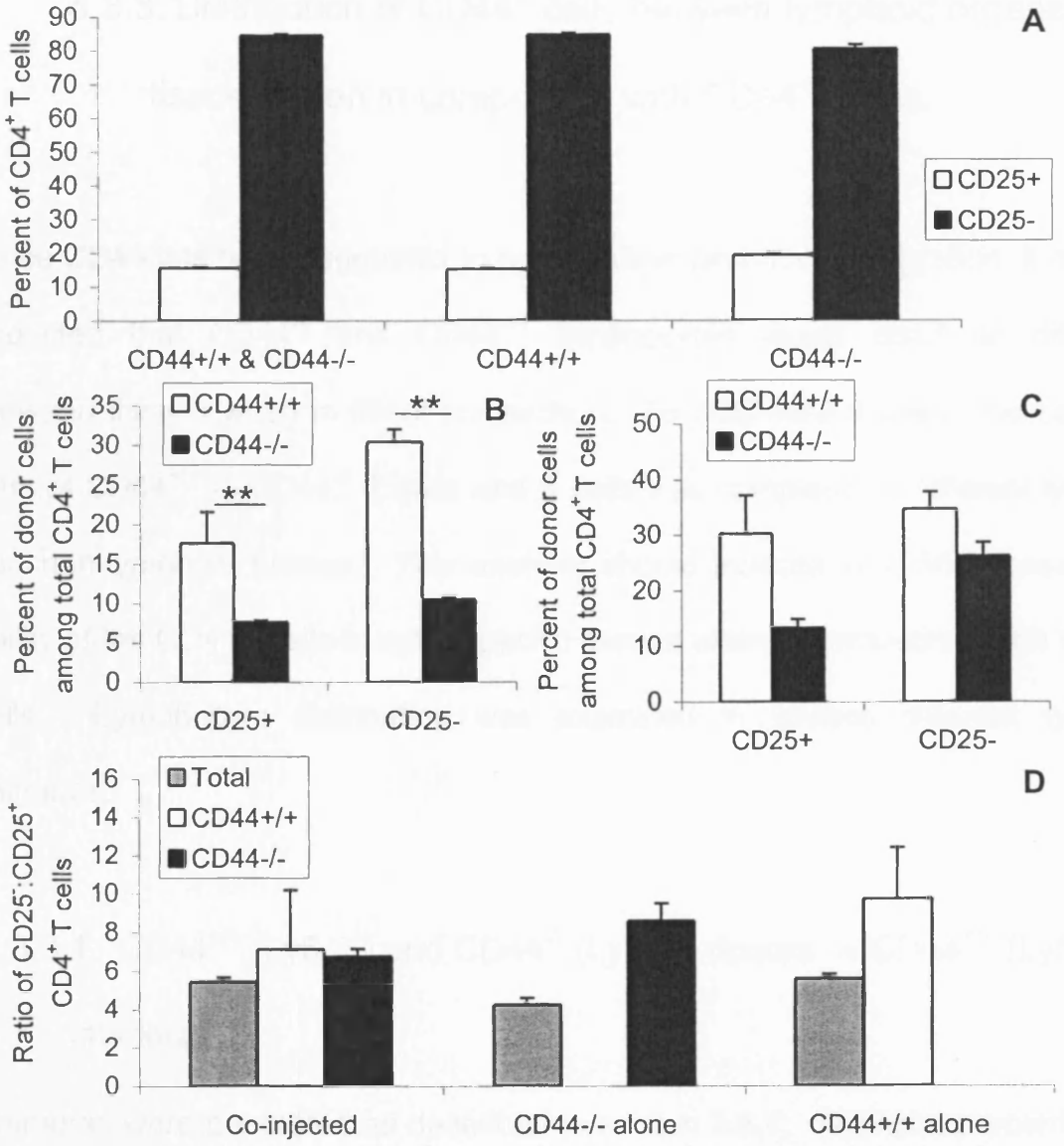

Figure 4.7. Analysis of $CD25^+$ and $CD25^-$ $CD4^+$ T cells 6 weeks after intrathymic injection of progenitor cells

A. Percent of total $CD25^+$ and $CD25^-$ $CD4^+$T cells in the spleen. B. Percent of donor $CD44^{+/+}$ and donor $CD44^{-/-}$ cells in each splenic $CD4^+$ T cell subset from mice co-injected with both $CD44^{+/+}$ and $CD44^{-/-}$ cells. C. Percent of donor $CD44^{+/+}$ and donor $CD44^{-/-}$ cells in each splenic $CD4^+$ T cell subset from mice injected with either $CD44^{+/+}$ or $CD44^{-/-}$ cells. D. The $CD25^-CD4^+$:$CD25^+CD4^+$ T cell ratio among total, and $CD44^{+/+}$ and $CD44^{-/-}$ donor populations. The data are mean ± s.e.m from groups of 5. P values, 2-sample t test; **, $P<0.01$.

4.3.3. Distribution of $CD44^{-/-}$ cells between lymphoid organs and tissues when in competition with $CD44^{+/+}$ cells.

Since CD44 has been suggested to participate in lymphocyte migration, it might be expected that $CD44^{-/-}$ and $CD44^{+/+}$ lymphocytes would distribute differently between tissues when in direct competition. To determine if this is the case, the ratio of $CD44^{+/+}$ to $CD44^{-/-}$ T cells and B cells was compared in different lymphoid and non-lymphoid tissues. This analysis should indicate any differences in the ability of the $CD44^{-/-}$ cells to enter specific tissues when in competition with $CD44^{+/+}$ cells. Lymphocyte distribution was examined in several different types of chimeras.

4.3.3.1. $CD44^{+/+}$ ($Ly5.2^{+}$) and $CD44^{-/-}$ ($Ly5.1^{+}$) donors $\rightarrow$ $CD44^{+/+}$ ($Ly5.2^{+}$) chimeras.

Chimeras were generated as described in section 3.3.1. The total percentages of $CD44^{+/+}$ and $CD44^{-/-}$ cells among $CD3^{+}$ and $CD19^{+}$ cells were compared to determine whether there was a difference in T and B cell distribution between the spleen and the LN. A significantly higher proportion of $CD44^{-/-}$ $CD19^{+}$ cells was found in pooled LN than in the spleen of these chimeras. There also appeared to be more $CD44^{+/+}$ $CD3^{+}$ cells in the spleen than the LN of these chimeras, although this was not significant (Figure 4.8A & B). A lower proportion of $CD44^{-/-}$ B cells was observed in peyer's patches compared to all other tissues examined.

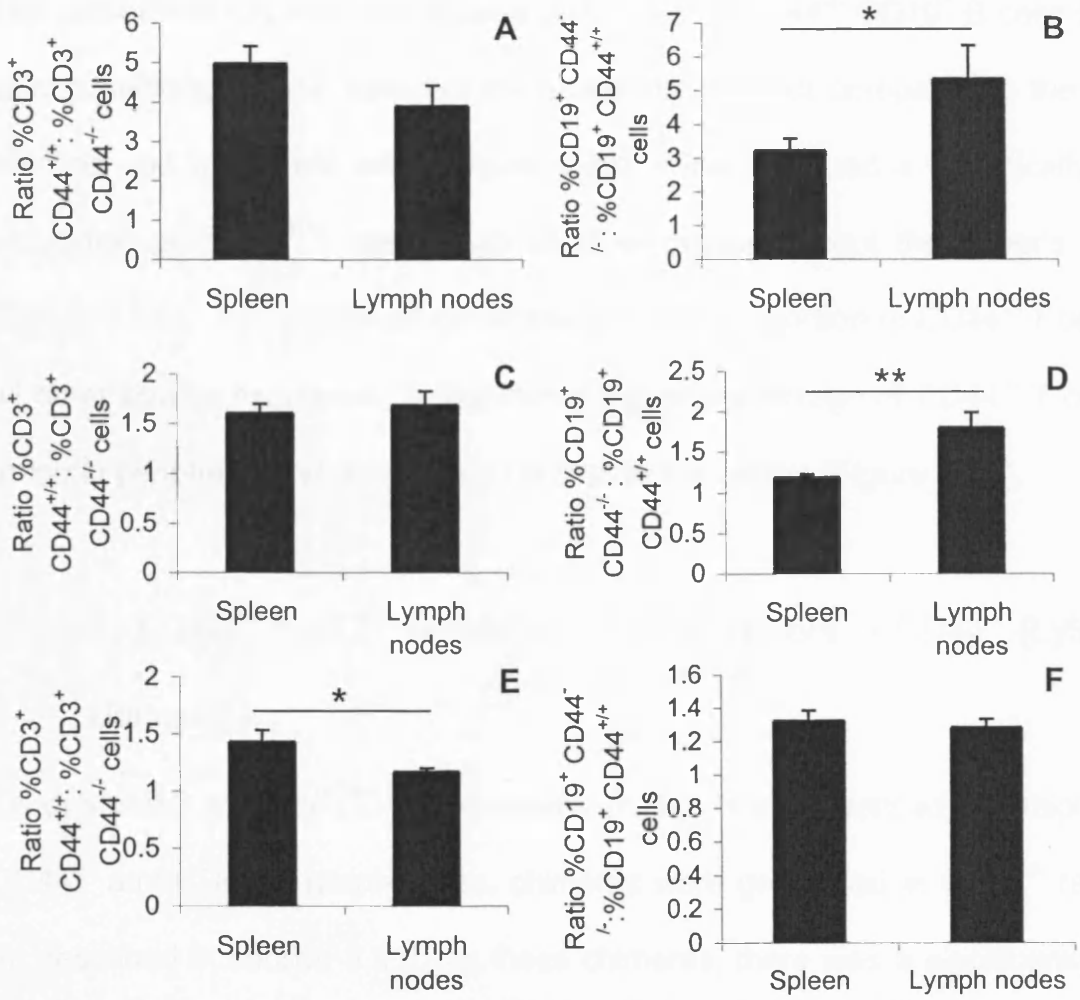

Figure 4.8. Analysis of $CD44^{+/+}$ vs. $CD44^{-/-}$ $CD3^+$ and $CD19^+$ cell ratios in spleen and lymph nodes of various chimeras.

A, B. Data obtained from 3 groups of 3 and a group of 4 $CD44^{+/+}$ ($Ly5.2^+$) & $CD44^{-/-}$ ($Ly5.1^+$) → $CD44^{+/+}$ ($Ly5.2^+$) chimeras. C, D. Data obtained from 2 groups of 4 and a group of 3 $CD44^{+/+}$ ($Ly5.2^+$) & $CD44^{-/-}$ ($Ly5.1^+$) → $CD44^{-/-}$ ($Ly5.1^+$). E, F. Data obtained from 1 groups of 3 and a group of 2 $CD44^{+/+}$ ($Ly5.1^+$ $Ly9.1^-$) & $CD44^{-/-}$ ($Ly5.1^+$ $Ly9.1^+$) → 1100rads $CD44^{+/+}$ ($Ly5.2^+$ Ly9.1). The data are mean ± s.e.m. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$.

The mesenteric LN also had a lower proportion of $CD44^{-/-}$ $CD19^{+}$ B cells than the spleen, although these were not as statistically relevant compared to the Peyer's patches and peritoneal cells (Figure 4.9B). The liver had a statistically higher proportion of $CD44^{-/-}$ T cells, than all other organs except the Peyer's patches (Figure 4.9A). The peritoneal cavity had a higher proportion of $CD44^{-/-}$ T cells than all other tissues examined. In addition a higher percentage of $CD44^{-/-}$ T cells was found in peripheral and mesenteric LN than in the spleen (Figure 4.9A).

4.3.3.2. $CD44^{+/+}$ ($Ly5.2^{+}$) and $CD44^{-/-}$ ($Ly5.1^{+}$) donors → $CD44^{-/-}$ ($Ly5.1^{+}$) chimeras.

To determine whether CD44 expression by the host influenced the migration of $CD44^{-/-}$ and $CD44^{+/+}$ lymphocytes, chimeras were generated in $CD44^{-/-}$ recipients as described in section 3.3.2. In these chimeras, there was a significantly higher proportion of $CD44^{-/-}$ $CD19^{+}$ cells in the LN than in the spleen, while there was a similar proportion of $CD44^{+/+}$ $CD3^{+}$ cells in the spleen and the LN (Figure 4.8C & D).

These results suggest that the difference in the ability of the $CD44^{-/-}$ B cells to enter the lymph node or spleen is independent of whether CD44 is expressed in the stromal environment, for example on the vascular walls.

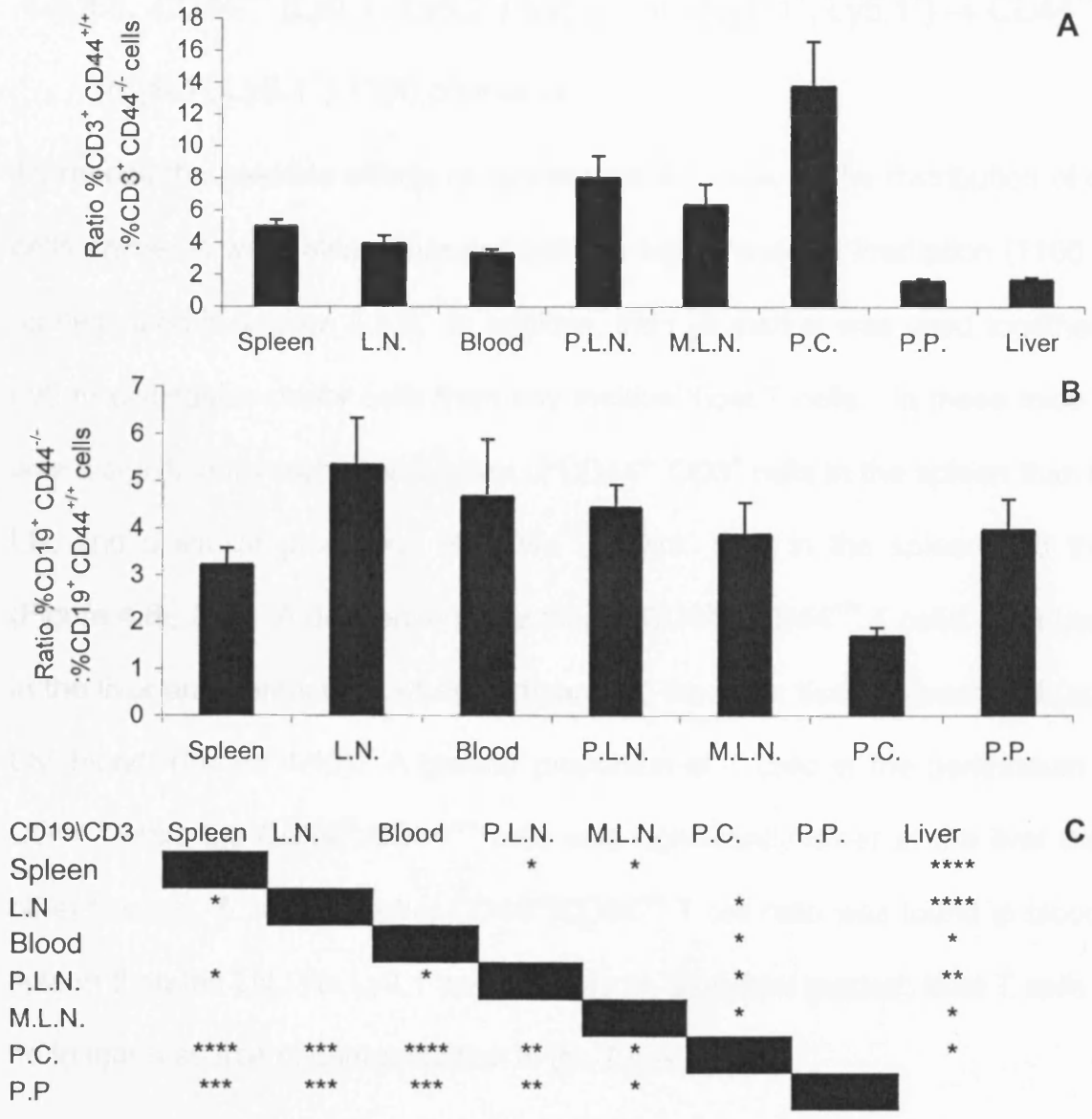

Figure 4.9. Analysis of $CD44^{+/+}$ vs. $CD44^{-/-}$ $CD3^+$ and $CD19^+$ cell ratios in various tissues of $CD44^{+/+}$ ($Ly5.2^+$) & $CD44^{-/-}$ ($Ly5.1^+$) → $CD44^{+/+}$ ($Ly5.2^+$) chimeras.

A. Ratio of percentage $CD3^+CD44^{+/+}$:$CD3^+CD44^{-/-}$ in various tissues.

B. Ratio of percentage $CD19^+CD44^{-/-}$:$CD3^+CD44^{+/+}$ in various tissues.

C. Table of P values of Figures B & C, $CD3^+$ statistics are horizontal, $CD19^+$ statistics are vertical. The data are mean ± s.e.m from groups between 4 and 8. L.N., total lymph nodes. P.L.N. peripheral lymph nodes. M.L.N. mesenteric lymph nodes. P.C. peritoneal cavity. p.p. Peyer's Patches. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$.

4.3.3.3. $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) and $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.1^+$) 1100 chimeras

To reduce the possible effects of radioresistant T cells on the distribution of donor cells chimeras were also generated using a higher dose of irradiation (1100 rads) as described in section 3.3.6. In addition, the Ly9 marker was used together with Ly5 to distinguish donor cells from any residual host T cells. In these mice there was a significantly higher proportion of $CD44^{-/-}$ $CD3^+$ cells in the spleen than in the LN, and a similar proportion of $CD44^{+/+}$ $CD19^+$ cells in the spleen and the LN (Figure 4.8E & F). A difference in the ratio of $CD44^{-/-}$:$CD44^{+/+}$ T cells was observed in the liver and peritoneum when compared to the other tissues examined (spleen, LN, blood) (Figure 4.10). A greater proportion of T cells in the peritoneum were $CD44^{-/-}$ while the $CD44^{-/-}$:$CD44^{+/+}$ ratio was significantly lower in the liver than all other tissues. A slightly higher $CD44^{-/-}$:$CD44^{+/+}$ T cell ratio was found in blood and spleen than the LN. As Ly9.1 was used as an analytical marker, host T cells were no longer a source of contamination in the T cell ratios.

4.3.3.4. x$CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) and y$CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.1^+$) chimeras.

To determine whether the distribution of $CD44^{-/-}$ lymphocytes was influenced by their relative frequency compared to $CD44^{+/+}$ cells, chimeras were generated using different ratios of $CD44^{-/-}$ and $CD44^{+/+}$ bone marrow as described in section 3.3.8. Ly9 and Ly5 antibodies were used to distinguish donor and recipient cells. In chimeras made at all ratios, a significantly higher proportion of $CD44^{-/-}$ $CD19^+$ cells was observed in the LN than the spleen.

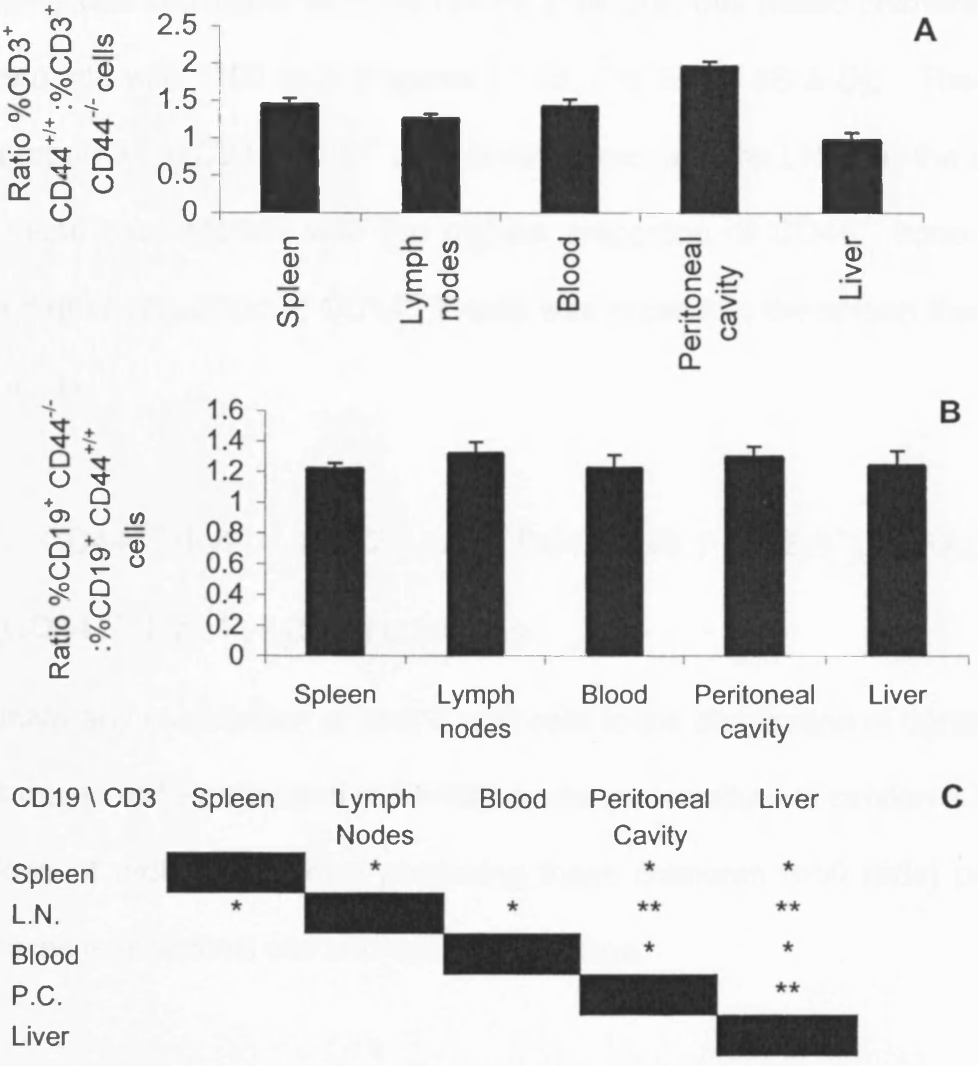

Figure 4. 10. Analysis of $CD44^{+/+}$ vs. $CD44^{-/-}$ $CD3^+$ and $CD19^+$ cell ratios in various tissues of $CD44^{+/+}$ ($Ly5.2^+$) & $CD44^{-/-}$ ($Ly9.1^+$) → $CD44^{+/+}$ ($Ly5.1^+$, $Ly9.1^-$) 1100 rads chimeras.

A. Ratio of percentage $CD3^+CD44^{+/+}$:$CD3^+CD44^{-/-}$ in various tissues.
B. Ratio of percentage $CD19^+CD44^{-/-}$:$CD3^+CD44^{+/+}$ in various tissues.
C. Table of P values of Figures B & C, $CD3^+$ statistics are horizontal, $CD19^+$ statistics are vertical. L.N., total lymph nodes. P.C. peritoneal cavity. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$. The data are mean ± s.e.m from 1 group of 3.

This finding was consistent with the results in all previous mixed chimeras except the mice made with 1100 rads (Figures 4.11B, D & E & 4.8B & D). There was a similar proportion of $CD44^{-/-}$ $CD3^{+}$ cells in the spleen and the LN of all the chimeras except those reconstituted with the highest proportion of $CD44^{-/-}$ bone marrow, where a higher proportion of $CD44^{-/-}$ T cells was present in the spleen than the LN (Figure 4.11).

4.3.3.5. $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) and $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras

To eliminate any contribution of host T or B cells to the distribution of donor derived cells, chimeras were generated in $RAG2^{-/-}$ hosts as described in section 3.3.7. The lower dose of radiation used in producing these chimeras (650 rads) potentially also induces less stromal cell and vascular damage.

In these mice there was a significantly higher proportion of $CD44^{-/-}$ $CD3^{+}$ cells in the spleen than in the LN consistent with observations in some of the other chimeras. However, unlike in other chimeras proportion of $CD44^{+/+}CD19^{+}$ cells was found in the spleen and LN (Figure 4.12A & B).

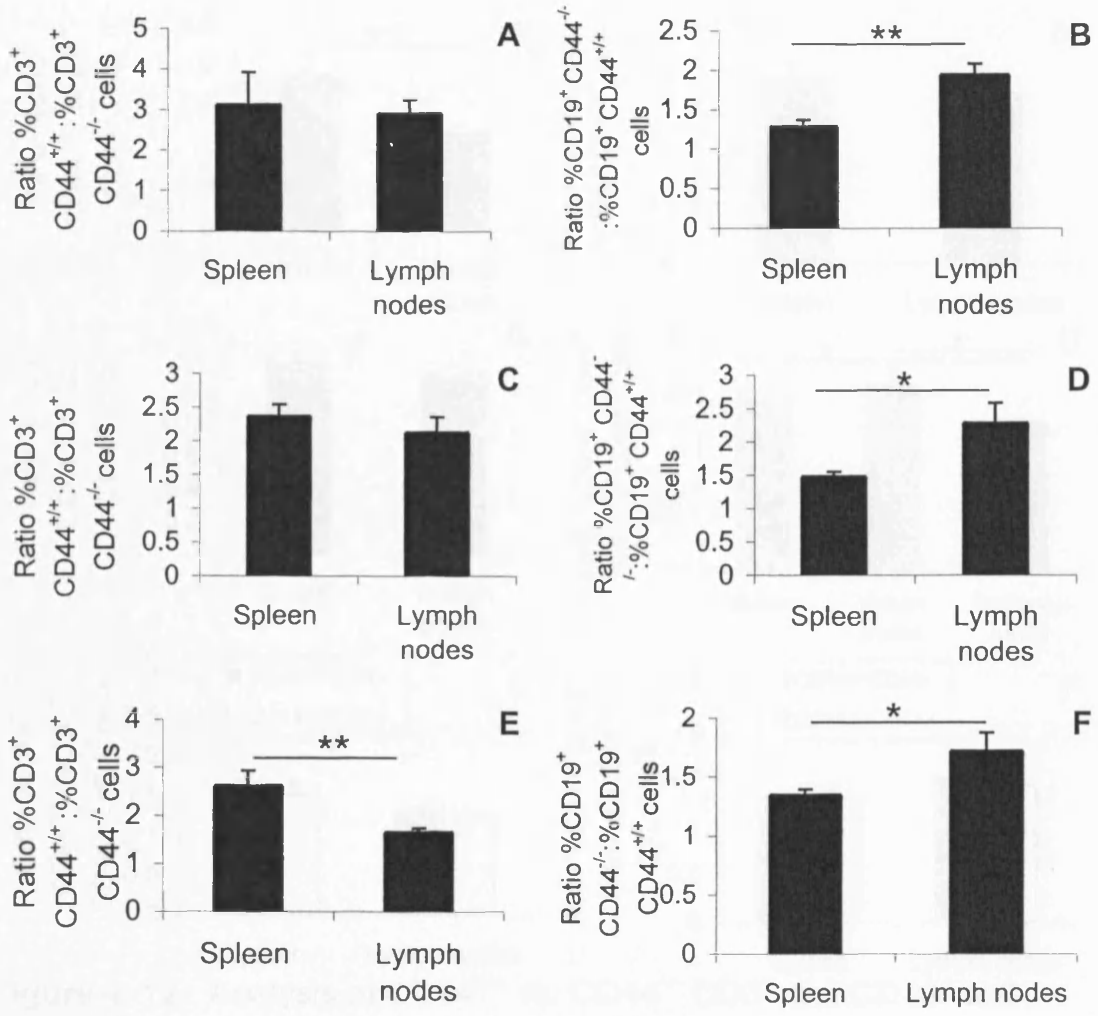

Figure 4. 11. Analysis of $CD44^{+/+}$ vs. $CD44^{-/-}$ $CD3^+$ and $CD19^+$ cell ratios in various tissues of $xCD44^{+/+}$ & $yCD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A & B, Data obtained from 1 group of 8 chimeras reconstituted with 25% $CD44^{-/-}$ bone marrow cells. C & D, Data obtained from 1 group of 4 chimeras reconstituted with 50% $CD44^{-/-}$ bone marrow cells. E & F, Data obtained from 1 group of 6 chimeras reconstituted with 75% $CD44^{-/-}$ bone marrow cells. The data are mean ± s.e.m. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$.

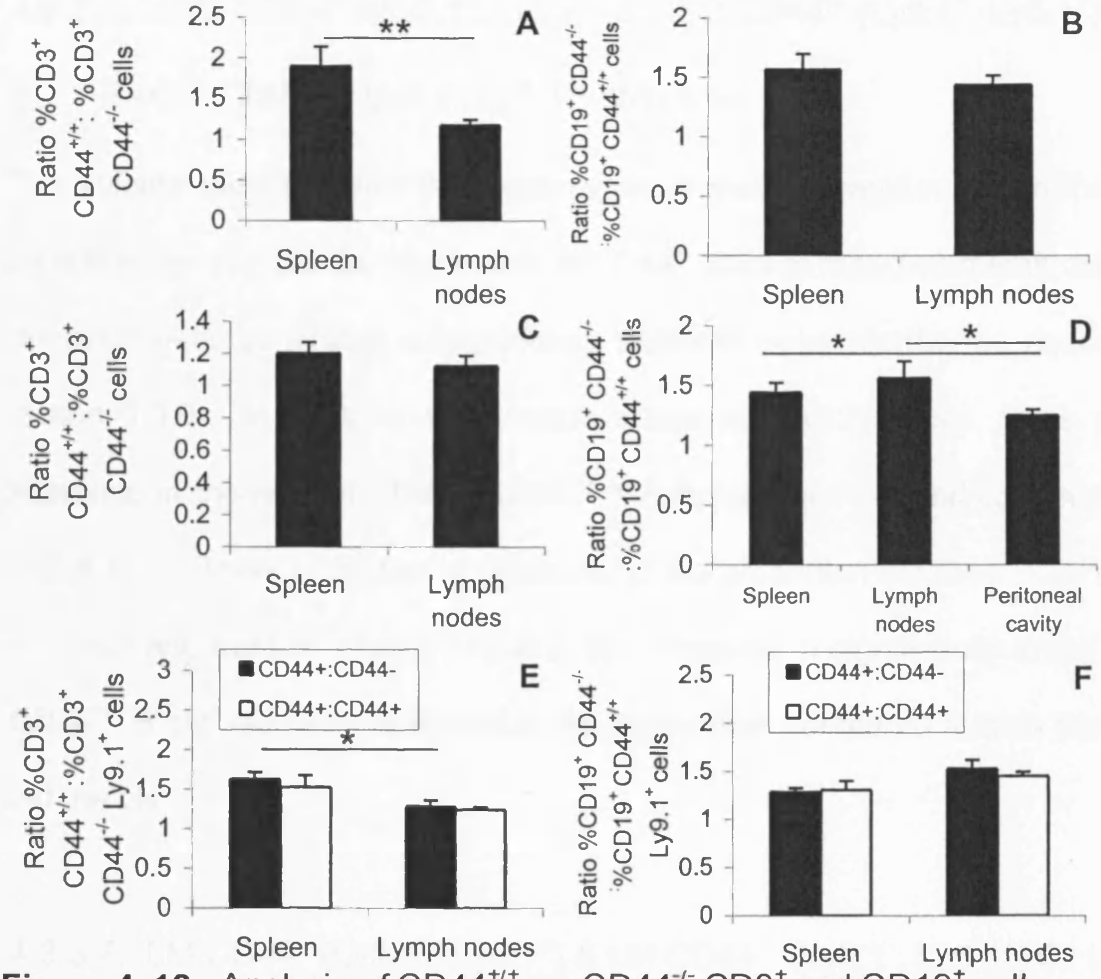

Figure 4. 12. Analysis of $CD44^{+/+}$ vs. $CD44^{-/-}$ $CD3^+$ and $CD19^+$ cell ratios in various tissues of various chimeras.

A & B, data obtained from 1 group of 2 and 2 groups of 3 $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) $\rightarrow$ $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras. C & D, data obtained from 1 group of 2 and 2 groups of 3 HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) $\rightarrow$ $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras. E & F, data obtained from 2 groups of chimeras: 1 group of 8 and 1 group of 9LM $CD44^+$ ($Ly9.1^+$, $Ly5.1^+$) & LM $CD44^{-/-}$ ($Ly9.1^-$, $Ly5.1^+$) / Or LM $CD44^+$ ($Ly9.1^+$, $Ly5.1^+$) & LM $CD44^+$ ($Ly9.1^-$, $Ly5.1^+$) & $\rightarrow$ $CD44^{+/+}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.2^+$) chimeras. The data are mean $\pm$ s.e.m.

4.3.3.6. HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras.

To eliminate the possibility that contaminating mature lymphocytes in the donor bone marrow affected the distribution of $CD44^{-/-}$ cells in mixed chimeras, additional chimeras were generated using lineage depleted bone marrow as described in section 3.3.9. In contrast to chimeras made in $RAG2^{-/-}$ hosts, there was no difference in the ratio of $CD44^{-/-}$:$CD44^{+/+}$ T cells between LN and spleen (Figures 4.12 A & C). There was also no difference in the proportion of $CD44^{+/+}$ $CD19^+$ cells in spleen and the LN (Figure 4.12 B & D). However, a significantly lower $CD44^{-/-}$:$CD44^{+/+}$ B cell ratio was observed in the peritoneum compared to both the spleen and the LN.

4.3.3.7. LM $CD44^+$ ($Ly9.1^+$, $Ly5.1^+$) & LM $CD44^{-/-}$ ($Ly9.1^-$, $Ly5.1^+$) Or LM $CD44^+$ ($Ly9.1^-$, $Ly5.1^+$) → $CD44^{+/+}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.2^+$) chimeras.

To eliminate any possible affects of differences in strain background, littermate (LM) chimeras were generated as described in section 3.3.10. A significantly higher proportion of $CD44^{-/-}$ T cells was found in the spleen than the LN of mixed chimeras (Figure 4.12 E). However, no differences in the differences in the distribution of $CD44^{-/-}$ B cells were detected. No differences were seen in the distribution of $CD44^+$ $Ly9.1^+$ vs. $Ly9.1^-$ cells in the control chimeras.

4.3.4. Adhesion profiles indicate subtle differences between $CD44^{+/+}$ and $CD44^{-/-}$ mice.

The previous section demonstrated that there were differences between $CD44^{+/+}$ and $CD44^{-/-}$ T and B cells in their propensity to migrate into secondary and tertiary lymphoid tissues. Since adhesion molecules other than CD44 participate in lymphocyte migration, the expression profile of other adhesion markers was investigated. $CD44^{+/+}$ and $CD44^{-/-}$ T and B cells were compared in the LN and spleens of two sets of chimeras.

4.3.4.1. Adhesion profiles of T and B cells from $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) and $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

Chimeras were generated as described in section 3.3.7. T and B cells from the spleen and LN were isolated and a range of adhesion markers was investigated. As described below, subtle differences in expression were observed.

4.3.4.1.1. $CD19^{+}$ cells:

$CD44^{-/-}$ B cells had an increased expression of CD62L, as assessed by both overall mean fluorescence intensity (MFI), and the MFI of the $CD62L^{hi}$ cells. There were also more $CD62L^{hi}$ $CD44^{-/-}$ cells than $CD44^{+/+}$ splenic B cells. $CD44^{-/-}$ B cells CD48 had slightly lower overall CD48 expression than $CD44^{+/+}$ cells, although a higher MFI was observed for the $CD48^{hi}$ $CD44^{-/-}$ B cells in both the spleen and LN. $CD19^{+}$ $CD44^{-/-}$ cells of the spleen and LN had lower expression of $\alpha_4$integrin, CD103, and LPAM1 than $CD44^{+/+}$ cells. In contrast there was higher expression of CD11b, CD54, CD48 and $\beta_2$integrin adhesion molecules on $CD44^{-/-}$ B cells in the

LN (Figures 4.13A, 4.14A, & 4.15A). Syndecan expression on sIgM^{lo} and sIgM^{hi} CD19^{+} CD44$^{+/+}$ and CD44$^{-/-}$ cells was also analysed in the spleen and lymph node, where CD44$^{-/-}$ B cells from both tissues displayed lower syndecan expression than CD44$^{+/+}$ cells in terms of overall MFI and the percentage of syndecan^{hi} cells (Figures 4.16 and 4.17).

4.3.4.1.2. T cells

There is a slight increase in the expression of CD11b, PECAM and CD102 in the spleen and CD11b and LPAM1 in the LN, as indicated by MFI, was observed for CD44$^{-/-}$ T cells when compared to CD44$^{+/+}$ T cells. A significant increase in the percentage of in CD11b^{hi}, and CD102^{hi} CD44$^{-/-}$ T cells in the spleen and CD11b^{hi}, CD102^{hi}, LPAM1^{hi} and $\beta_2$integrin^{hi} CD44$^{-/-}$ T cells in the LN was also apparent. In contrast there was a decrease in the percentage of CD54^{hi} CD44$^{-/-}$ T cells. Finally, there was an increase in the MFI of CD11a^{hi} and $\alpha_4$integrin^{hi} CD44$^{-/-}$ splenic T cells and of CD11a^{hi}, CD11b^{hi}, LPAM1^{hi} and $\alpha_4$integrin^{hi} lymph node CD44$^{-/-}$ T cells (Figures 4.18, 4.19, & 4.20).

4.3.4.1.3. Co-expression of integrins

T and B cells that were double positive for either $\alpha_4$integrin and LPAM1, or, $\beta_2$integrin and LPAM1, were also analysed. There was a reduction in the percent of CD44$^{-/-}$ cells found in $\beta_2$integrin and LPAM1 double positive B cells in both the spleen and LN compared to total CD19^{+} cells. CD44$^{-/-}$ T cells in the LN also had a reduction in the percentage of $\beta_2$integrin and LPAM1 double positive cells.

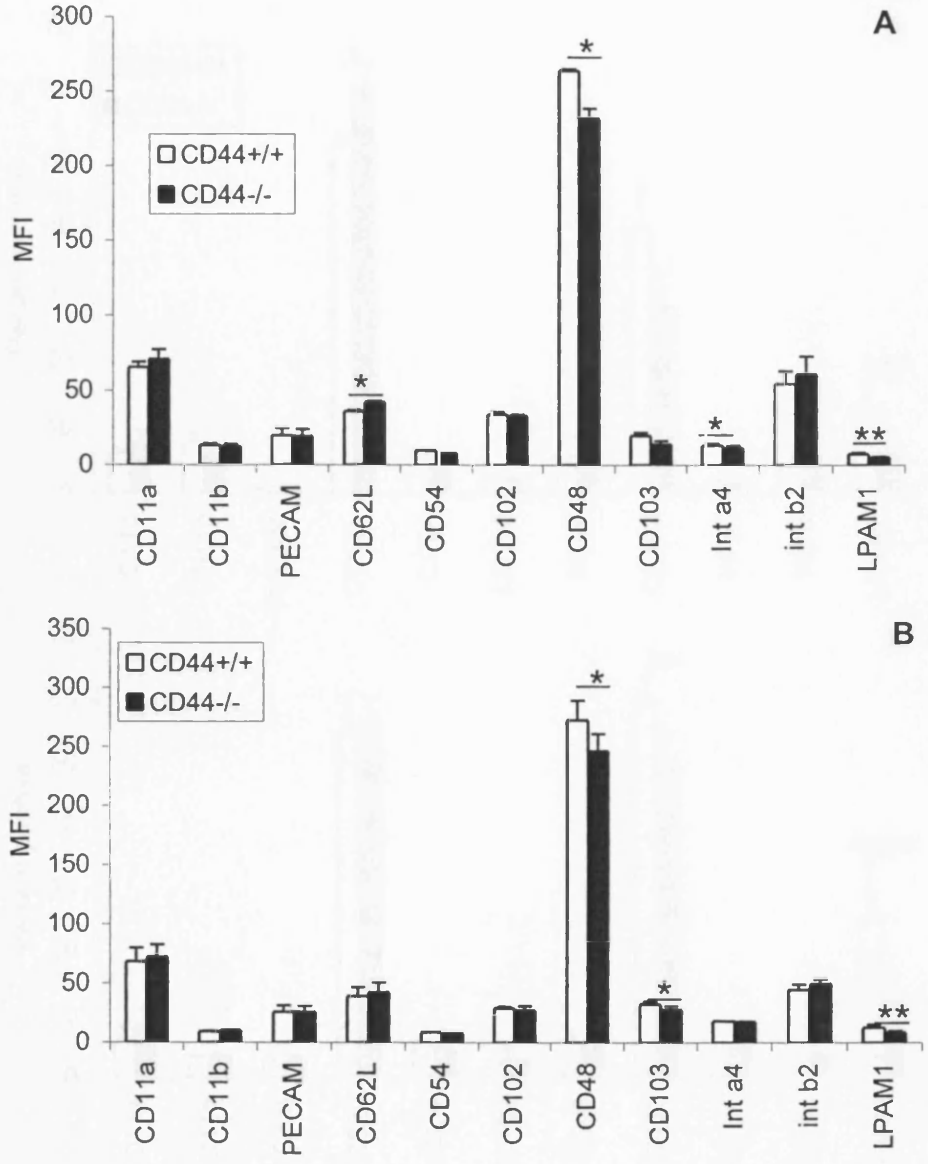

Figure 4.13. Expression of adhesion molecules in CD19^{+} cells in CD44$^{+/+}$ (Ly9.1^{-}, Ly5.2^{+}) & CD44$^{-/-}$ (Ly9.1^{+}, Ly5.1^{+}) $\rightarrow$ RAG2$^{-/-}$ (CD44$^{+/+}$, Ly9.1^{-}, Ly5.1^{+}) chimeras.

A. Adhesion profile comparing CD44$^{+/+}$ and CD44$^{-/-}$ CD19^{+} cells in spleen. B. Adhesion profile comparing CD44$^{+/+}$ and CD44$^{-/-}$ CD19^{+} cells in lymph nodes. The data show the MFI for total cells for each marker and are mean $\pm$ s.e.m. from groups of 3. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$.

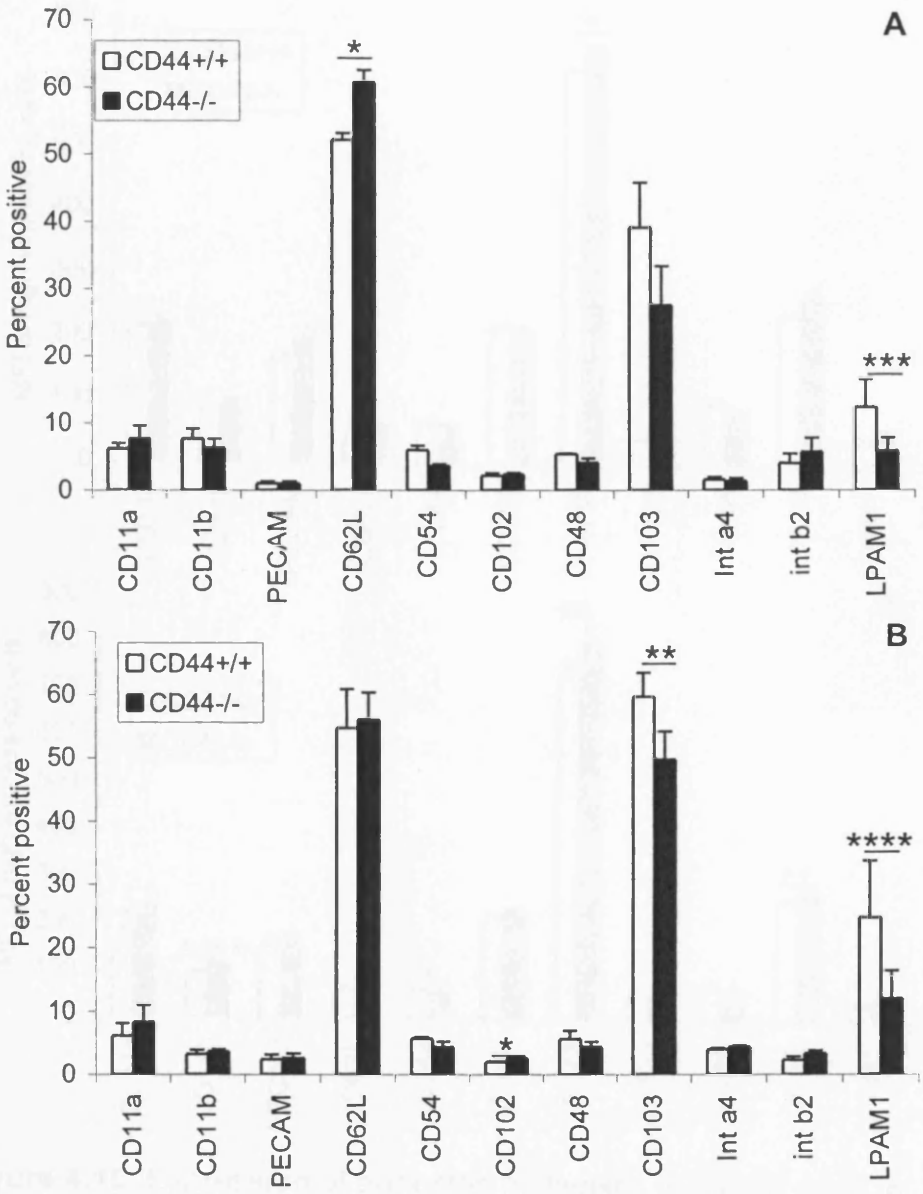

Figure 4.14. Expression of adhesion molecules in $CD19^+$ cells in $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras. II.

A. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD19^+$ cells in spleen. B. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD19^+$ cells in lymph nodes. The data show percent positive cells for each marker and are mean ± s.e.m. from groups of 3. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$.

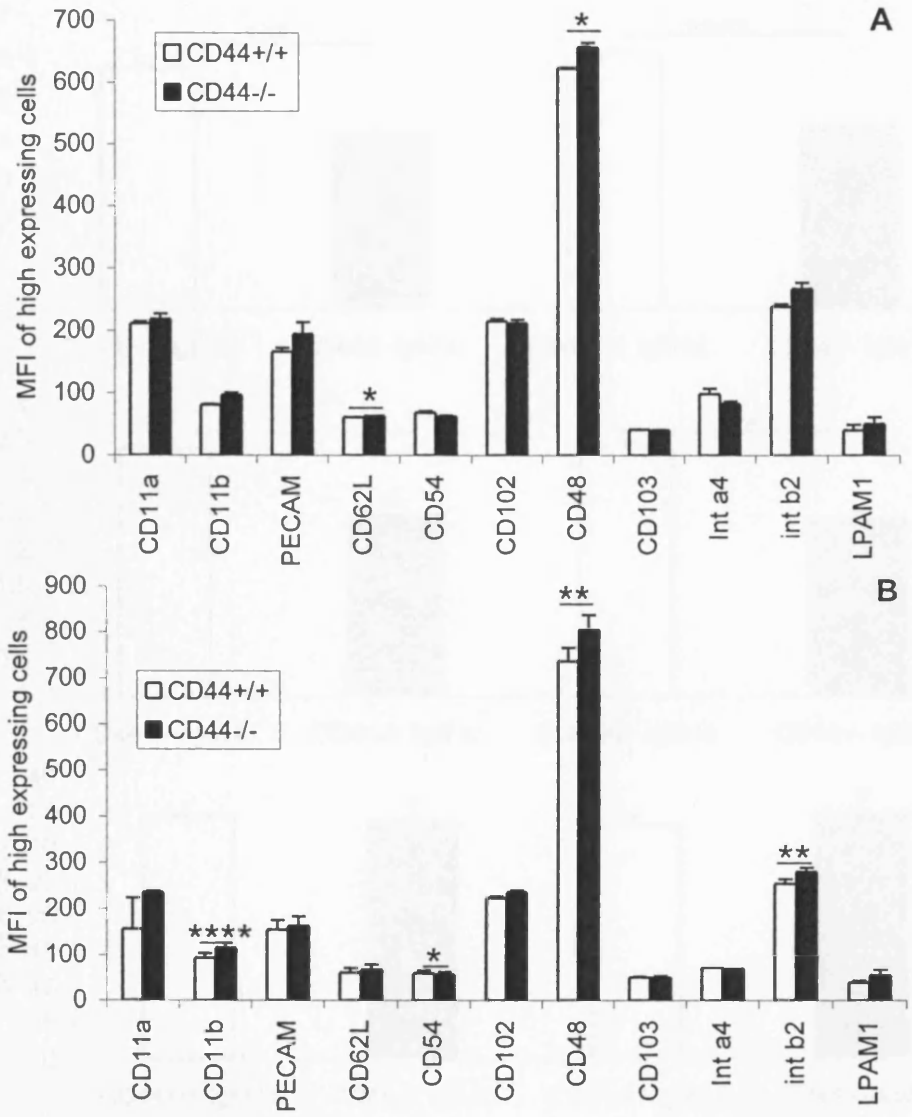

Figure 4.15. Expression of adhesion molecules in $CD19^+$ cells in $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) $\rightarrow$ $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras. III.

A. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD19^+$ cells in spleen. B. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD19^+$ cells in lymph nodes. The data shows MFI of cells gated for high expression of the indicated marker and are mean $\pm$ s.e.m. from groups of 3. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ****, $P<0.001$.

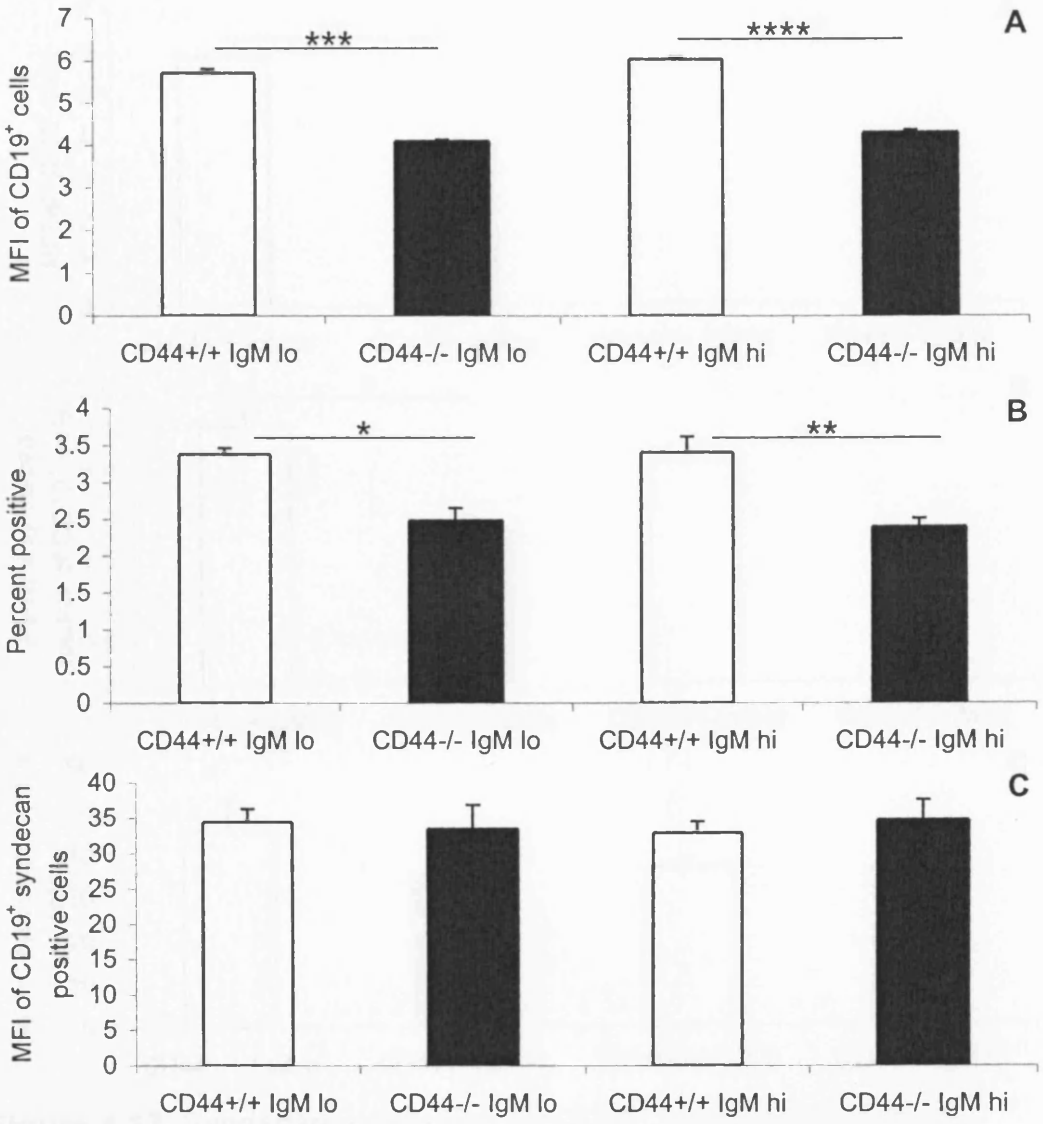

Figure 4.16. Syndecan expression of splenic $CD19^+$ cells in $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras.

A. Expression of syndecan (MFI for total cells) in $CD19^+$ $sIgM^{lo}$ or $sIgM^{hi}$ $CD44^{+/+}$ or $CD44^{-/-}$ donor cells. B. Percent syndecan positive $CD19^+$ $sIgM^{lo}$ or $sIgM^{hi}$ $CD44^{+/+}$ or $CD44^{-/-}$ donor cells. C. MFI of syndecan positive $CD19^+$ $sIgM^{lo}$ or $sIgM^{hi}$ $CD44^{+/+}$ or $CD44^{-/-}$ donor cells. The data are mean ± s.e.m from groups of 3. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$.

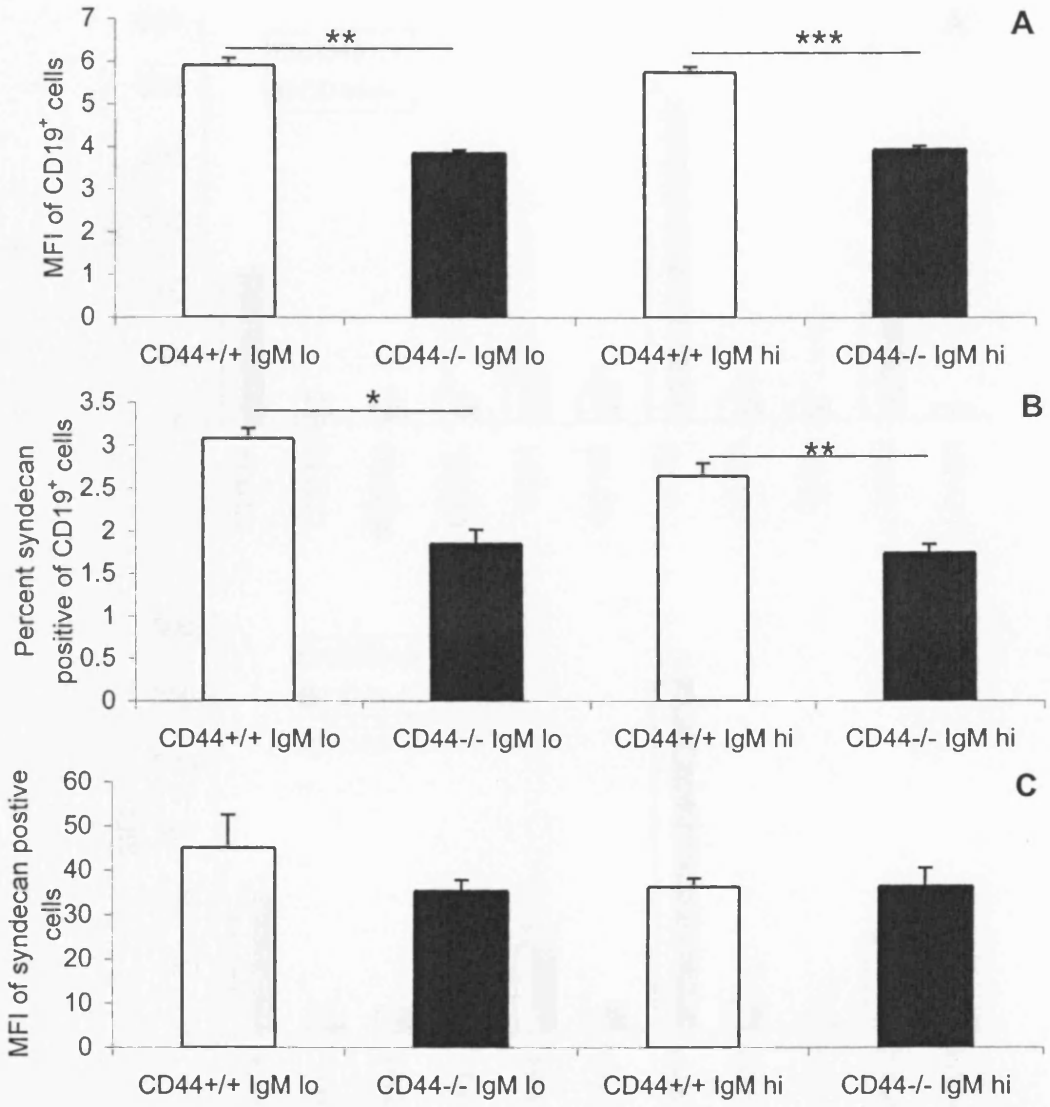

Figure 4.17. Syndecan expression on $CD19^+$ lymph nodes cells in $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras.

A. Expression of syndecan (MFI of total cells) on $CD19^+$ $sIgM^{lo}$ or $sIgM^{hi}$ $CD44^{+/+}$ or $CD44^{-/-}$ donor cells. B. Percent syndecan positive $CD19^+$ $sIgM^{lo}$ or $sIgM^{hi}$ $CD44^{+/+}$ or $CD44^{-/-}$ donor cells. C. MFI of syndecan positive $CD19^+$ $sIgM^{lo}$ or $sIgM^{hi}$ $CD44^{+/+}$ or $CD44^{-/-}$ donor cells. The data are mean ± s.e.m from groups of 3. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$.

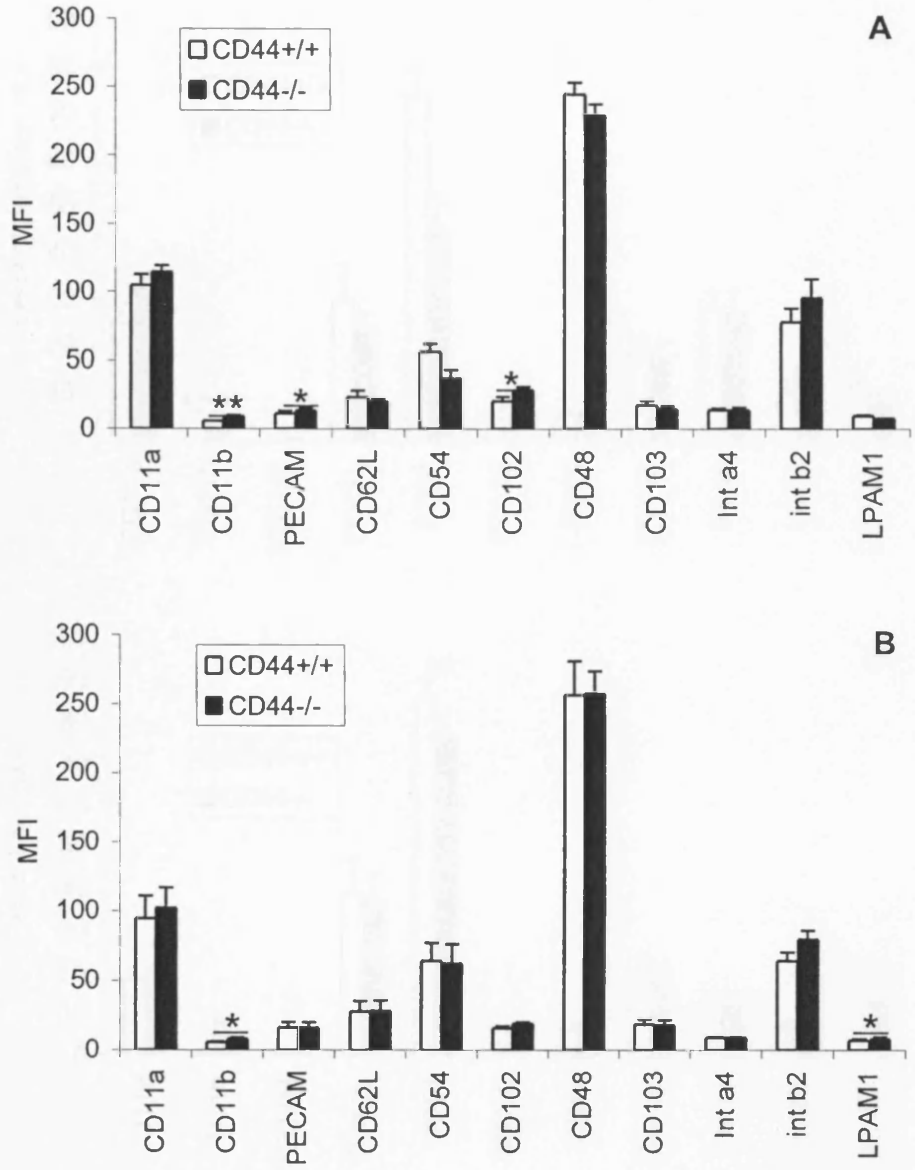

Figure 4.18. Expression of adhesion molecules in $CD3^+$ cells in $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras. I.

A. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD3^+$ cells in spleen. B. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD3^+$ cells in lymph nodes. The data shows the MFI for total cells for each marker and are mean ± s.e.m. from groups of 3. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$.

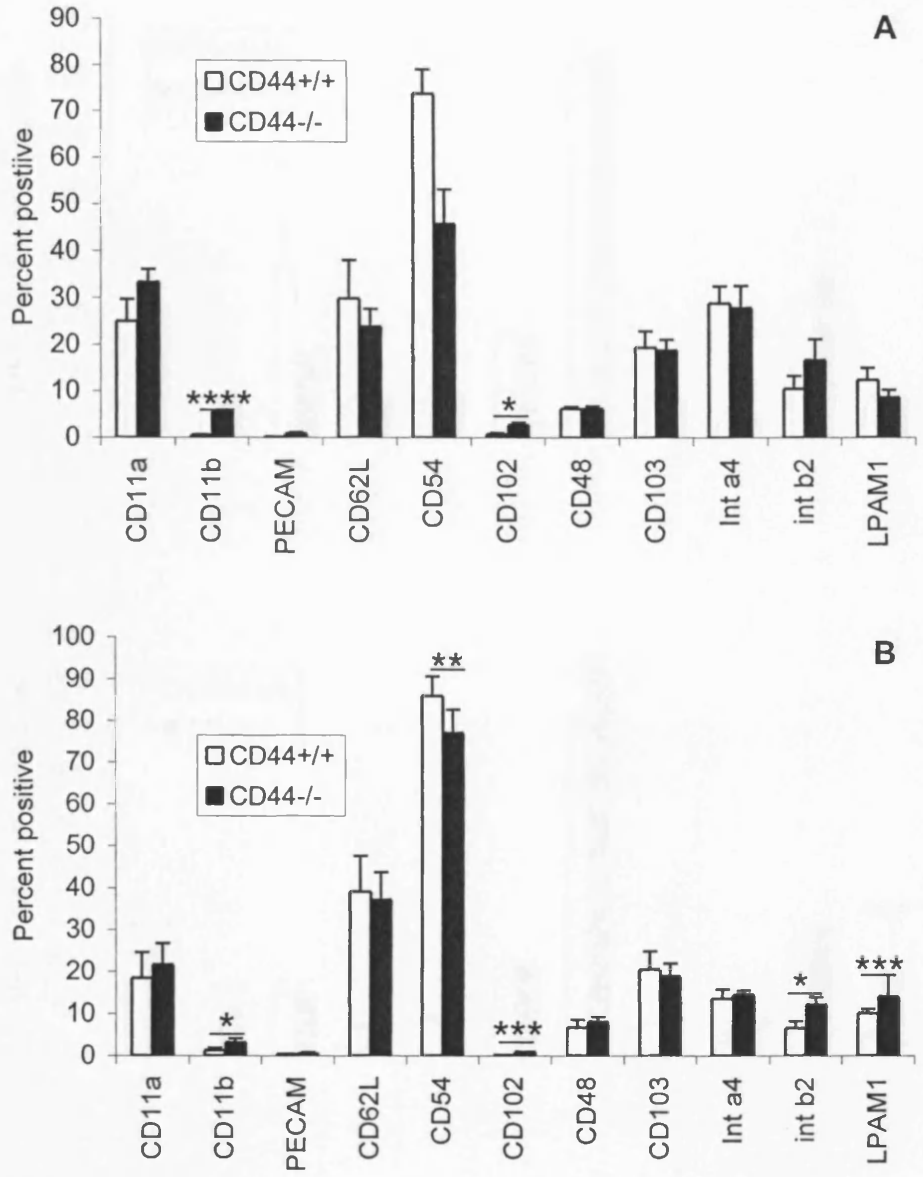

Figure 4.19. Expression of adhesion molecules on $CD3^+$ cells in $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras. II.

A. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD3^+$ cells in spleen. B. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD3^+$ cells in lymph nodes. The data shows the percent postive cells for each marker and are mean ± s.e.m. from groups of 3. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$.

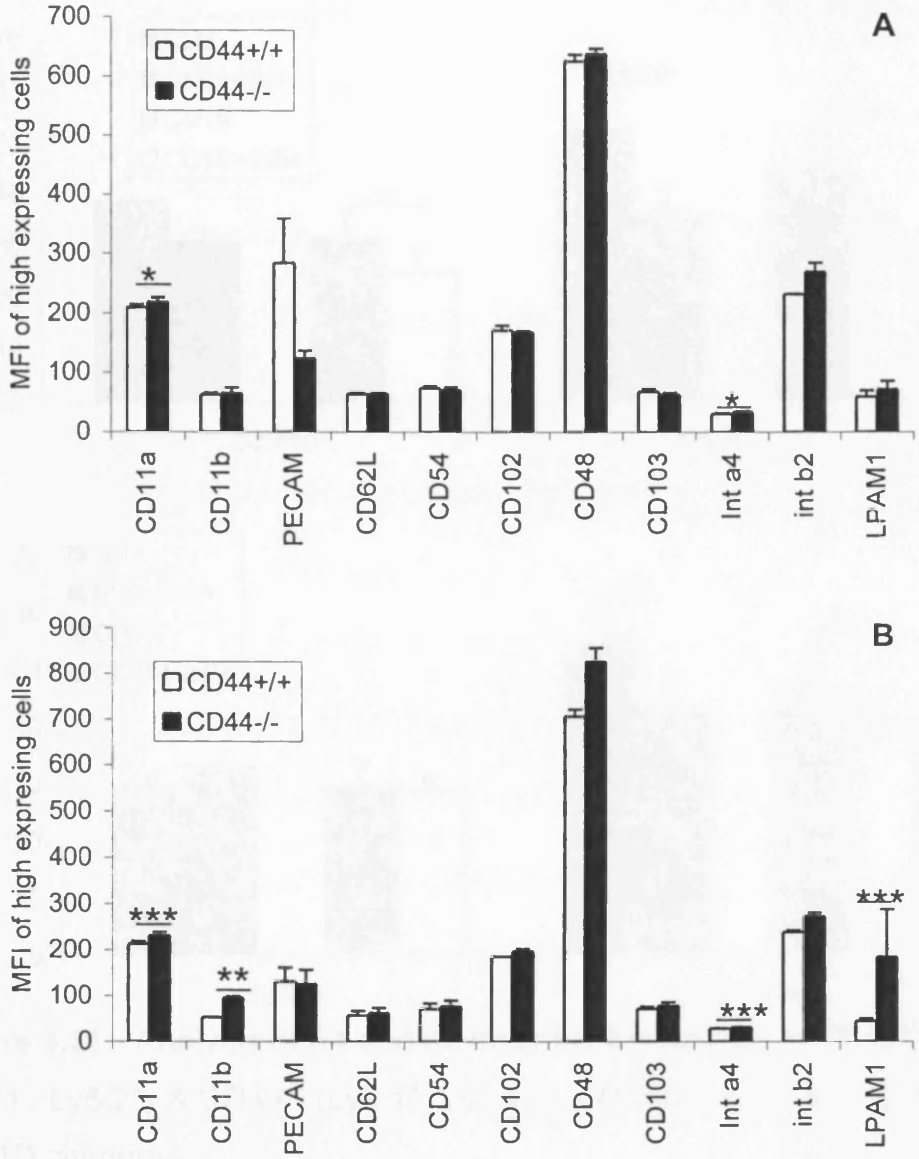

Figure 4.20. Expression of adhesion molecules on $CD3^+$ cells in $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras. III.

A. Adhesion profiles comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD3^+$ cells in spleen. B. Adhesion profiles comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD3^+$ cells in lymph nodes. The data show MFI for cells gated for high expression of the individual markers and are mean ± s.e.m. from groups of 3. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$.

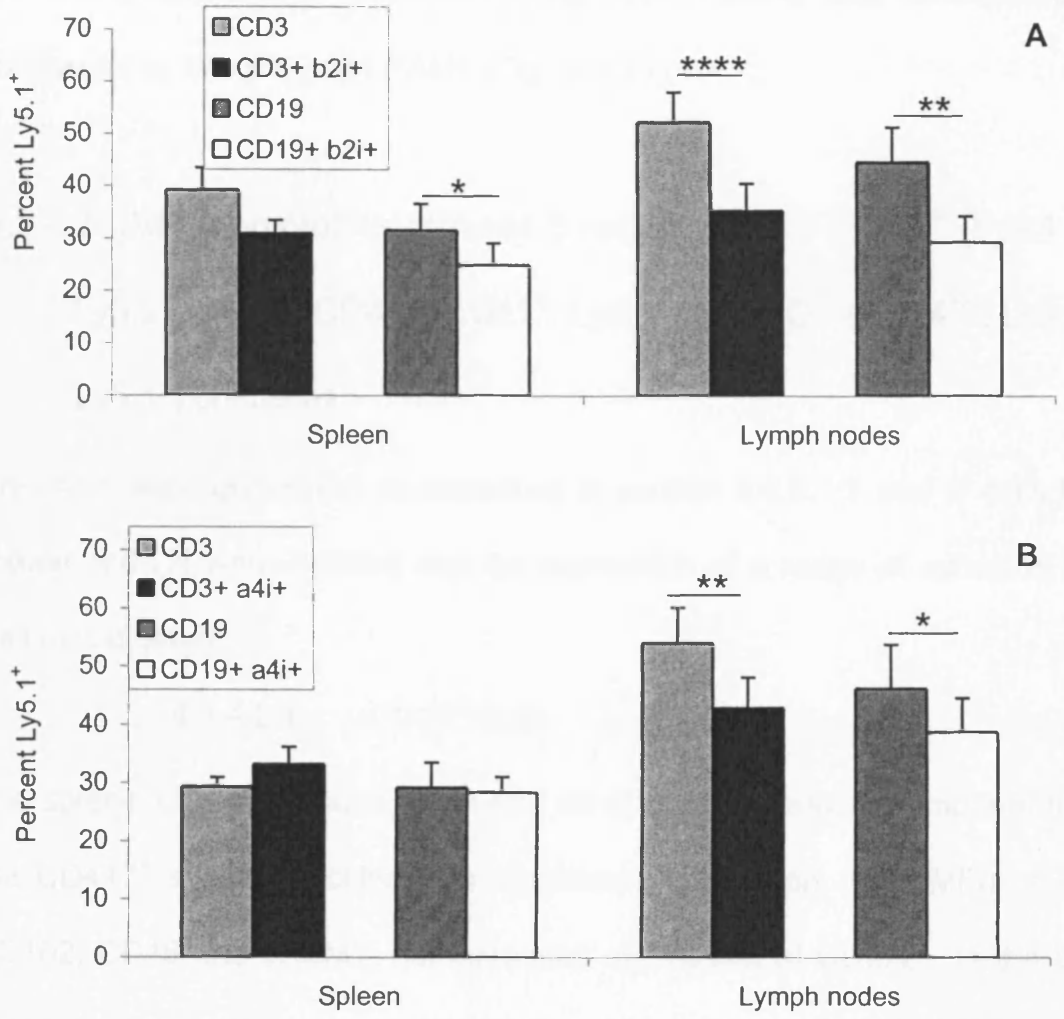

Figure 4.21. Analysis of $\alpha 4$ and $\beta 2$ integrins expression in $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) $\rightarrow$ $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

A. Percentage of $CD44^{-/-}$ ($Ly5.1^{+}$) cells found among total $CD3^{+}$ or $CD19^{+}$ populations compared to $CD3^{+}$ or $CD19^{+}$ $LPAM^{+}\beta_2integrin^{+}$ (b2i) cells. B. Percentage of $CD44^{-/-}$ ($Ly5.1^{+}$) cells found among total $CD3^{+}$ or $CD19^{+}$ populations compared to $CD3^{+}$ or $CD19^{+}$ $LPAM^{+}\alpha_4integrin^{+}$ (a4i) cells. The data are mean $\pm$ s.e.m. from groups of 3. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ****, $P<0.001$.

In the LN, there was a reduction among both T and B cells which were double positive for α_4 integrin and LPAM1 (Figure 4.21).

4.3.4.2. Adhesion profiles of T and B cells from HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras

Chimeras were generated as described in section 3.3.9. T and B cells from the spleen and LN were isolated and the expression of a range of adhesion markers was investigated.

4.3.4.2.1. $CD19^+$ cells

The splenic $CD44^{-/-}$ B cells, again had an altered phenotype compared to that of the $CD44^{+/+}$ splenic B cells, with decreased expression (total MFI) of PECAM, CD102, CD48 and LPAM1, but increased expression of CD62L. In the LN there was increased expression of CD11a and $\beta_2$integrin but decreased expression of CD102, CD48 and LPAM1. The percentage of cells expressing high levels of certain markers also differs between $CD44^{+/+}$ and $CD44^{-/-}$ splenic B cells, in that there was a decrease in the percentage of $CD44^{-/-}$ cells that were $CD54^{hi}$, $CD102^{hi}$, $CD48^{hi}$, $CD103^{hi}$, and $LPAM1^{hi}$, but an increased percentage of $CD44^{-/-}$ cells that were $CD62L^{hi}$ and $\alpha_4$integrin^{hi}. Among $CD19^+$ cells of the LN there was a decrease in the percentage of $CD44^{-/-}$ cells that were $CD11b^{hi}$, $CD102^{hi}$, $CD48^{hi}$, $CD103^{hi}$, $LPAM1^{hi}$, but an increased $CD44^{-/-}$ cell contribution $CD11a^{hi}$, $CD54^{hi}$, $\alpha_4$integrin^{hi}, and $\beta_2$integrin^{hi} populations. The MFI of the cells expressing high levels of different markers was also different between $CD44^{+/+}$ and $CD44^{-/-}$ splenic B cells. There

was an increased MFI among $CD44^{-/-}$ $CD62L^{hi}$, $CD102^{hi}$, and unusually $LPAM1^{hi}$ cells. In the LN there was an increased MFI $CD44^{-/-}$ B cells that were $CD11b^{hi}$, $CD62L^{hi}$, $CD48^{hi}$, $CD103^{hi}$, $\beta_2$integrin^{hi} and $LPAM1^{hi}$ (Figures 4.22, 4.23, 4.24). Syndecan expression on $sIgM^{lo}$ and $sIgM^{hi}$ $CD19^{+}$ $CD44^{+/+}$ and $CD44^{-/-}$ cells was also analysed in the spleen and lymph node, where $CD44^{-/-}$ B cells from both tissues displayed lowered syndecan expression in terms of overall MFI and the percentage of syndecan^{hi} cells, than $CD44^{+/+}$ B cells (Figures 4.25 and 4.26).

4.3.4.2.2. $CD3^{+}$ Cells

In the spleen, $CD44^{-/-}$ T cells, again had an altered phenotype to that of the $CD44^{+/+}$ T cells. There was increase expression (total MFI) of CD11a, CD11b and CD54 on the $CD44^{-/-}$ cells. In the LN there was an increased expression of CD11a, CD11b, CD54, CD48, CD103 and $\beta_2$integrin. There was also an increased percentage of cells expressing high levels of CD11a, CD11b, CD54, CD102, CD103, and $\beta_2$integrin adhesion molecules among $CD44^{-/-}$ splenic T cells. In the LN this was true for CD11a, CD54, CD48, CD103, $\beta_2$integrin. The MFI of the high expressing cells was also significantly different between $CD44^{+/+}$ and $CD44^{-/-}$ splenic T cells, with an increased MFI for $CD11a^{hi}$, $CD11b^{hi}$, $PECAM1^{hi}$, $CD54^{hi}$, $\alpha_4$integrin^{hi} and $LPAM1^{hi}$ $CD44^{-/-}$ cells. The MFI for $CD62L^{hi}$ $CD44^{-/-}$ splenic T cells was reduced compared to that for $CD62L^{hi}$ $CD44^{+/+}$ cells. In the LN there was an increased MFI for $CD44^{-/-}$ T cells expressing high levels of CD11a, CD11b, PECAM, CD62L, CD48, CD102, CD103, $\beta_2$integrin and LPAM1 (Figures 4.27-4.29).

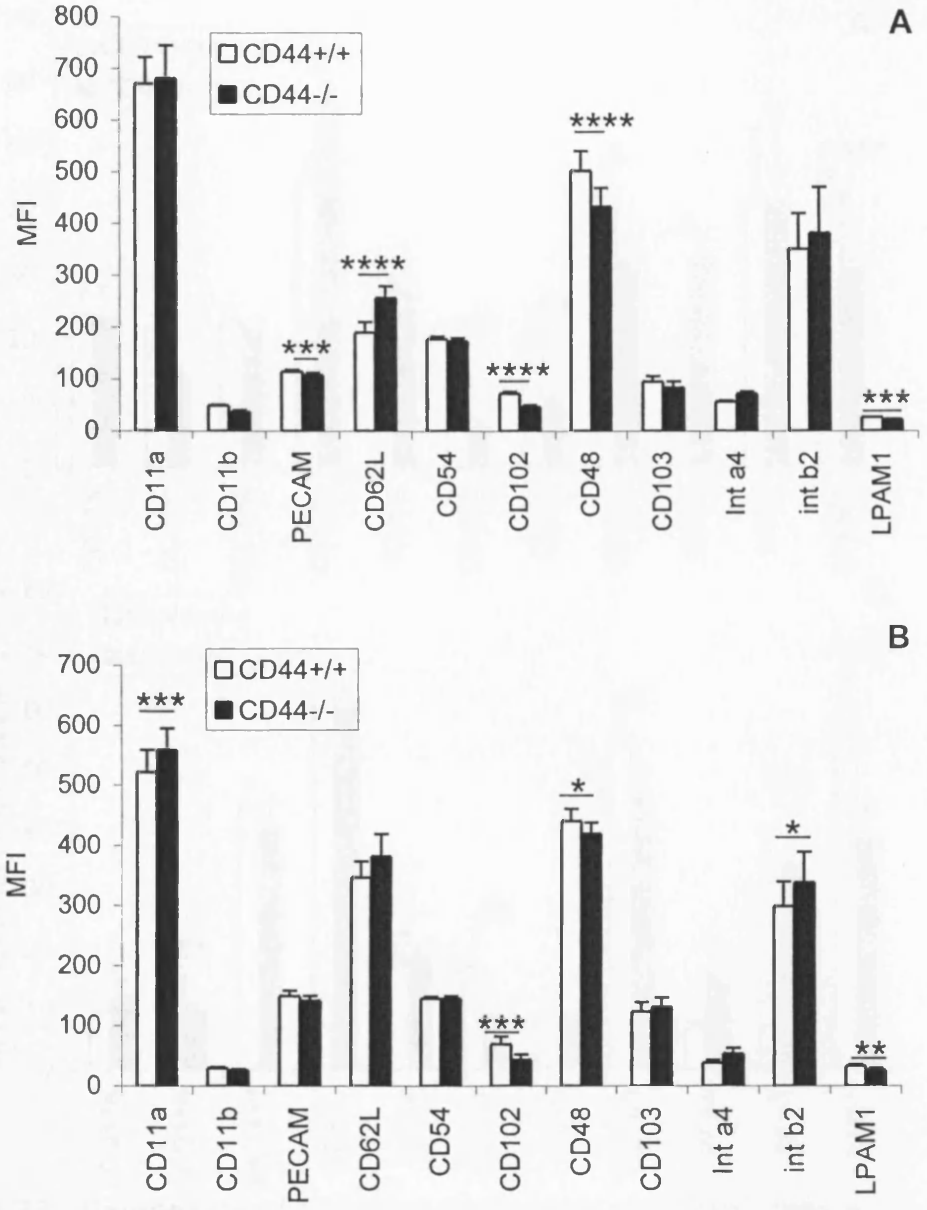

Figure 4.22. Expression of adhesion molecules on $CD19^+$ cells in HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) $\rightarrow$ $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras. I.

A. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD19^+$ cells in spleen. B. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD19^+$ cells in lymph nodes. The data shows the MFI for total cells for each marker and are mean $\pm$ s.e.m. from groups of 4. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$.

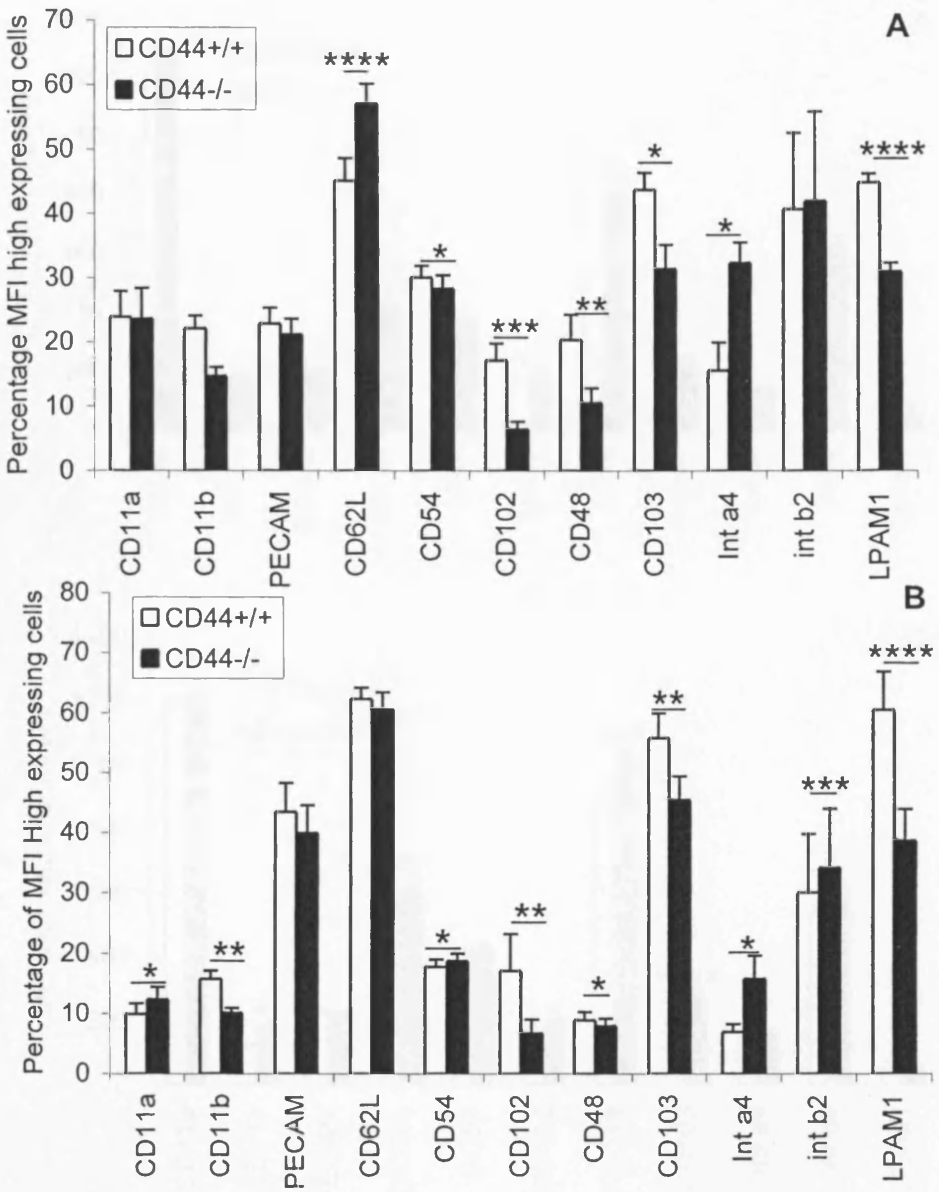

Figure 4.23. Expression of adhesion molecules on $CD19^+$ cells in HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras. II.

A. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD19^+$ cells in spleen. B. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD19^+$ cells in lymph nodes. The data shows the percent of cells expressing high levels of the indicated marker and are mean ± s.e.m. from groups of 4. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$.

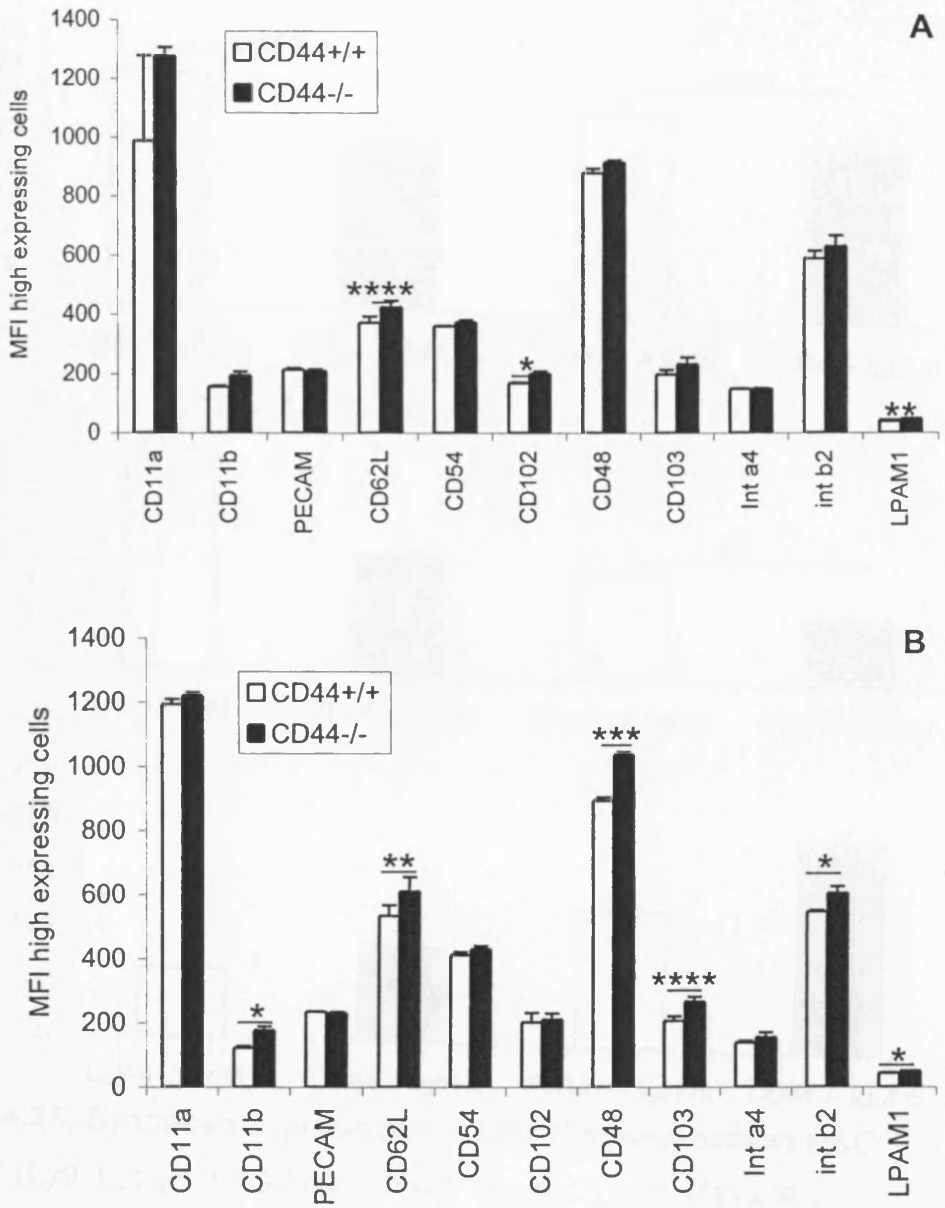

Figure 4.24. Expression of adhesion on $CD19^+$ cells in HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras. III.

A. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD19^+$ cells in spleen. B. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD19^+$ cells in lymph nodes. The data shows the MFI for cells expressing high levels of the indicated marker and are mean ± s.e.m. from groups of 4. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$.

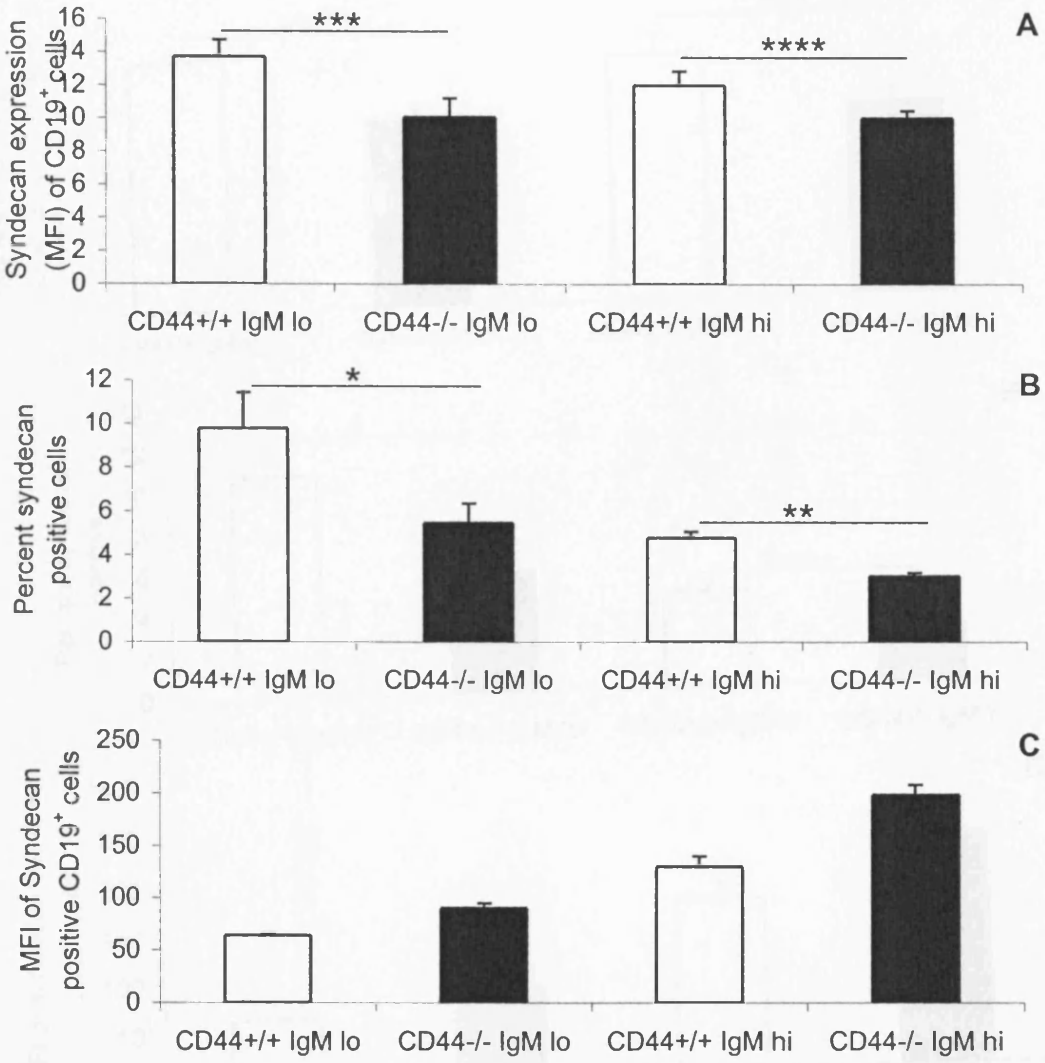

Figure 4.25. Syndecan expression on $CD19^+$ spleen cells in HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras.

A. Syndecan expression on $CD19^+$ $sIgM^{lo}$ or $sIgM^{hi}$ $CD44^{+/+}$ or $CD44^{-/-}$ donor cells. B. Percent syndecan positive of $CD19^+$ $sIgM^{lo}$ or $sIgM^{hi}$ $CD44^{+/+}$ or $CD44^{-/-}$ donor cells. C. MFI of syndecan positive $CD19^+$ $sIgM^{lo}$ or $sIgM^{hi}$ $CD44^{+/+}$ or $CD44^{-/-}$ donor cells. The data are mean ± s.e.m from groups of 4. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$.

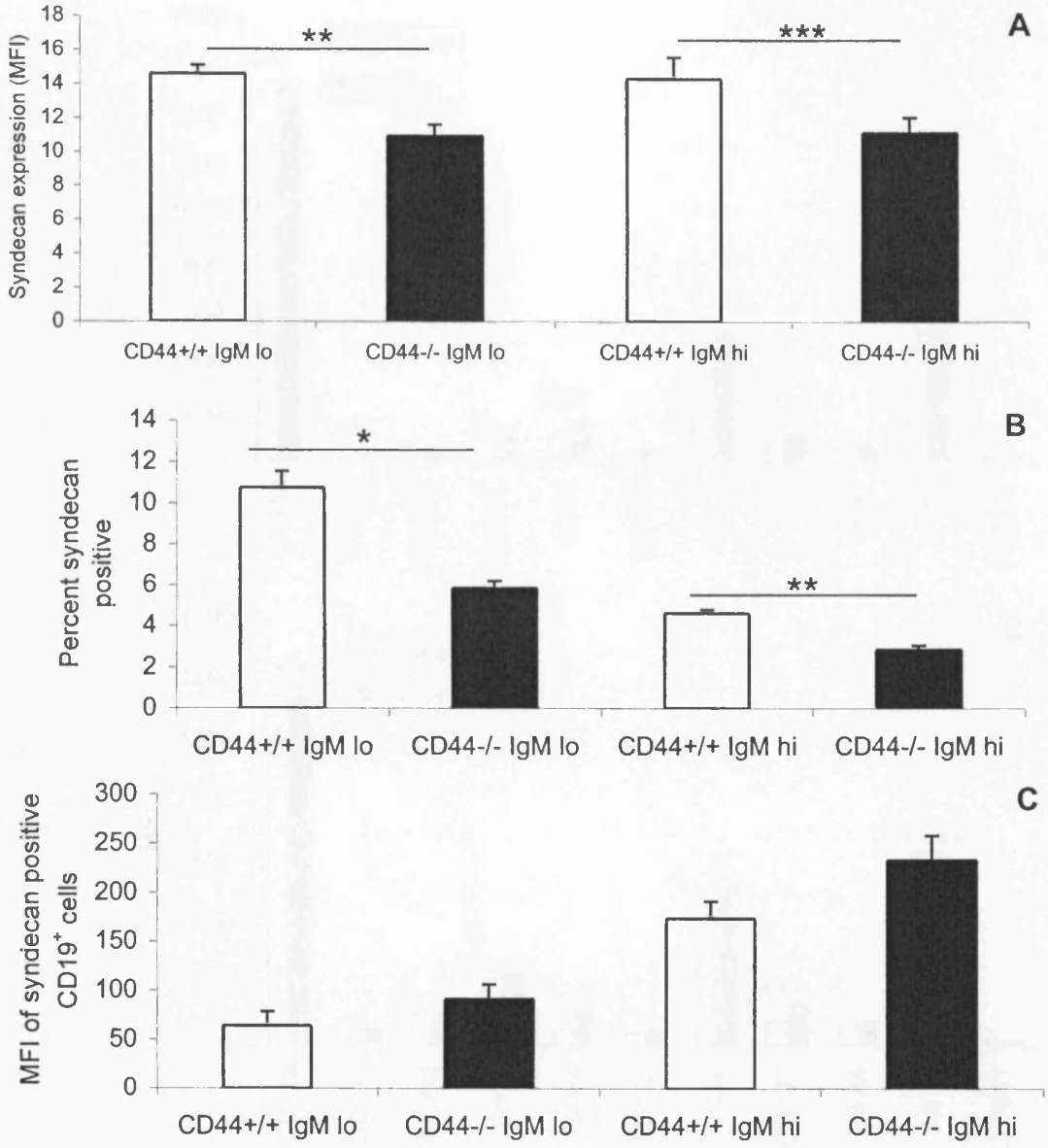

Figure 4.26. Syndecan expression on $CD19^+$ lymph nodes cells in HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras.

A. Syndecan expression on $CD19^+$ $sIgM^{lo}$ or $sIgM^{hi}$ $CD44^{+/+}$ or $CD44^{-/-}$ donor cells (MFI for total cells). B. Percent syndecan positive of $CD19^+$ $sIgM^{lo}$ or $sIgM^{hi}$ $CD44^{+/+}$ or $CD44^{-/-}$ donor cells. C. MFI of syndecan positive $CD19^+$ $sIgM^{lo}$ or $sIgM^{hi}$ $CD44^{+/+}$ or $CD44^{-/-}$ donor cells. The data are mean ± s.e.m from groups of 4. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$.

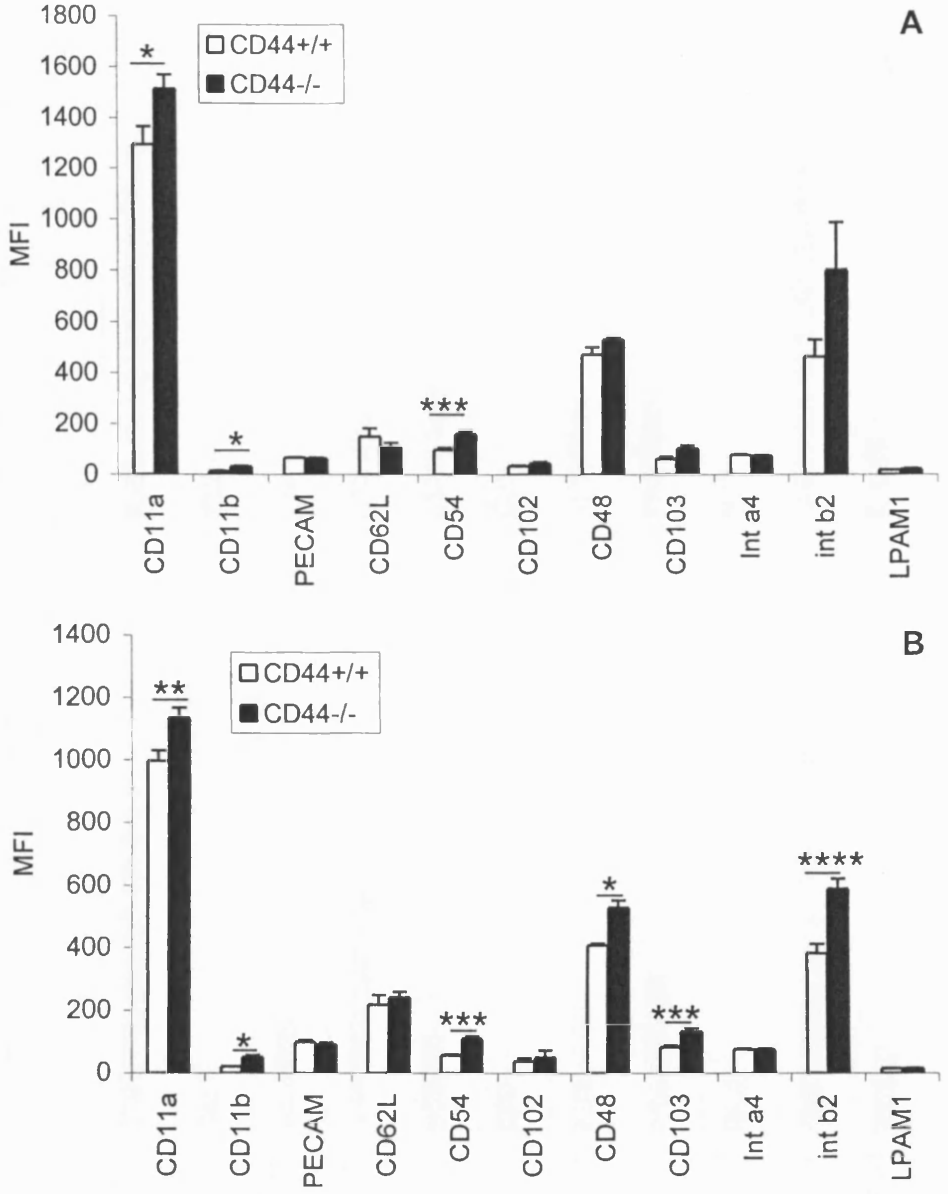

Figure 4.27. Expression of adhesion molecules on $CD3^+$ cells in HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras. I.

A. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD3^+$ cells in spleen. B. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD3^+$ cells in lymph nodes. The data shows MFI cells for total cells for each marker and are mean ± s.e.m. from groups of 4. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$.

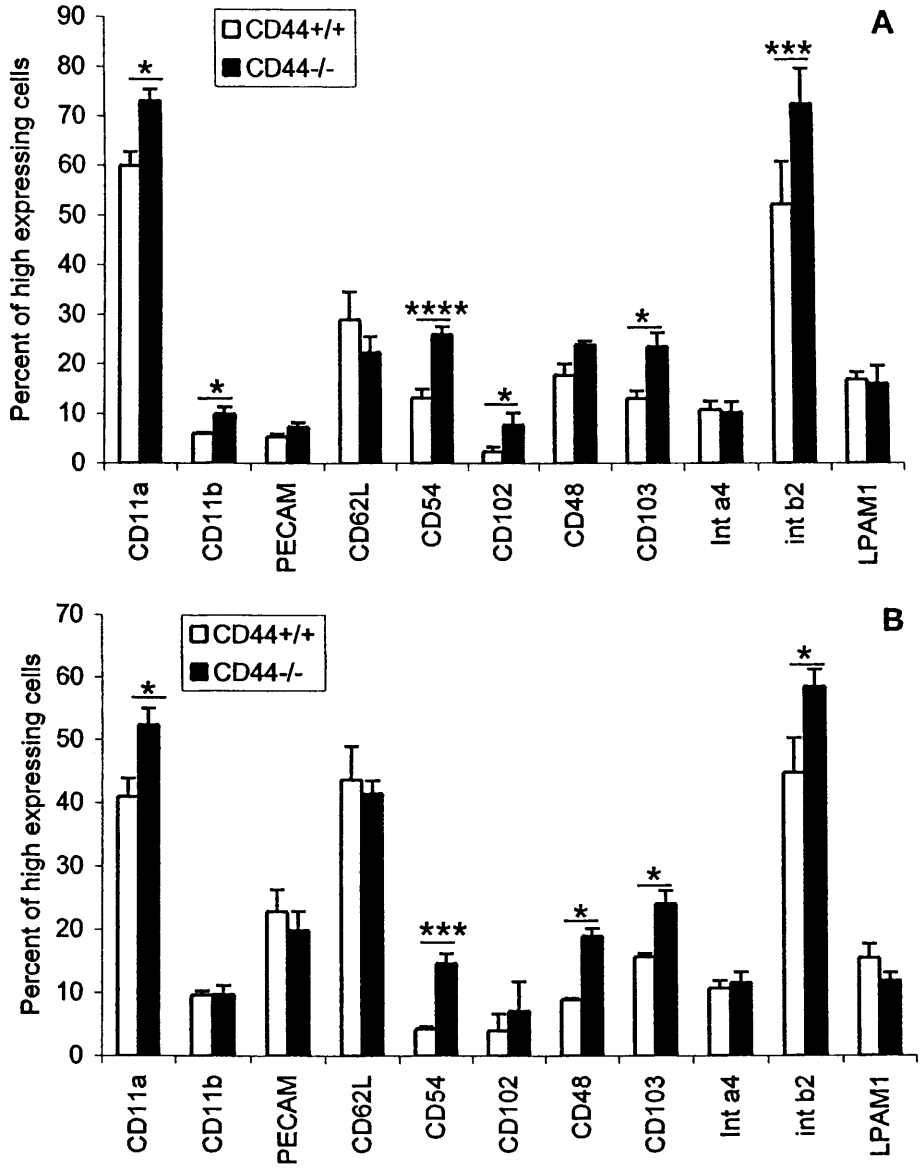

Figure 4.28. Expression of adhesion molecules on $CD3^+$ cells in HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras. II.

A. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD3^+$ cells in spleen. B. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD3^+$ cells in lymph nodes. The data shows the percentage of cells expressing high levels of the indicated marker and are mean ± s.e.m. from groups of 4. P values, 2-sample t test; *, $P<0.05$, ***, $P<0.005$, ****, $P<0.001$.

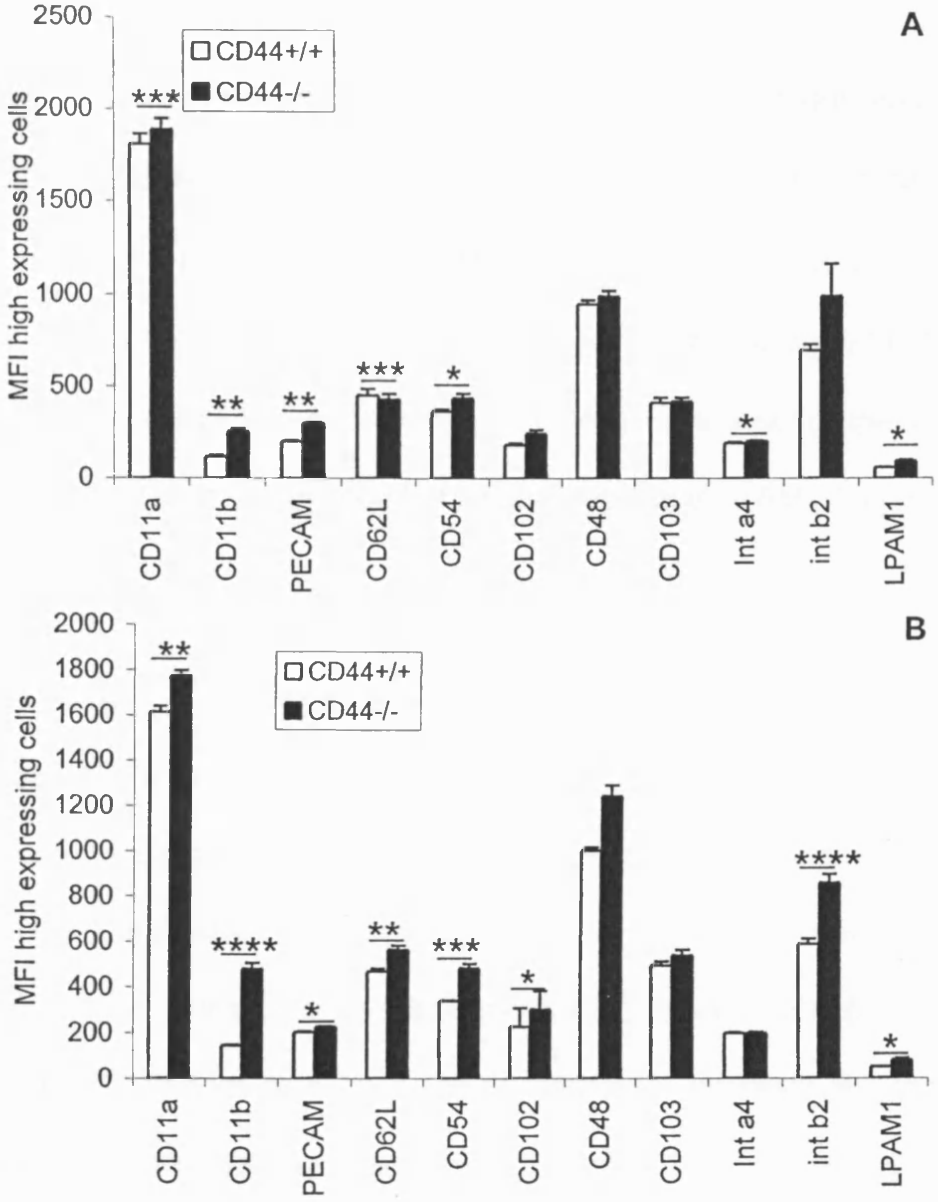

Figure 4.29. Expression of adhesion molecules on $CD3^+$ cells in HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras. III.

A. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD3^+$ cells in spleen. B. Adhesion profile comparing $CD44^{+/+}$ and $CD44^{-/-}$ $CD3^+$ cells in lymph nodes. The data shows MFI for cells gated on high expression of the indicated marker and are mean ± s.e.m. from groups of 4. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$.

4.3.4.2.3. Integrin double positive cells

The T and B cells that were double positive for either $\alpha_4$integrin and LPAM1, or, $\beta_2$integrin and LPAM1, were analysed. There was a reduced percentage of $CD44^{-/-}$ cells found among $\beta_2$integrin and LPAM1 double positive B cells in both the spleen and LN compared to the percentage of $CD44^{-/-}$ cells among total $CD19^{+}$ cells. There was also a reduced contribution of $CD44^{-/-}$ cells among spleen and lymph node T cells that were double positive for $\alpha_4$integrin and LPAM1 (Figure 4.30).

4.3.5. $CD44^{-/-}$ cells are still able to bind to hyaluronate.

We assessed the ability of $CD44^{-/-}$ cells to bind to the principle ligand of CD44, hyaluronate, focussing specifically on the ligand binding activity of dendritic cells (DCs). DCs were derived from either $CD44^{+/+}$ or $CD44^{-/-}$ bone marrow and were incubated with a range of concentrations of FITC labelled HA (FITC-HA) for 10 minutes at 4°C, before being thoroughly washed. Interestingly, there was no statistical difference between the amount of FITC-HA bound by $CD44^{-/-}$ and $CD44^{+/+}$ DCs, although the $CD44^{-/-}$ cells appeared to bind less FITC-HA at the lowest dilution (Figure 4.31).

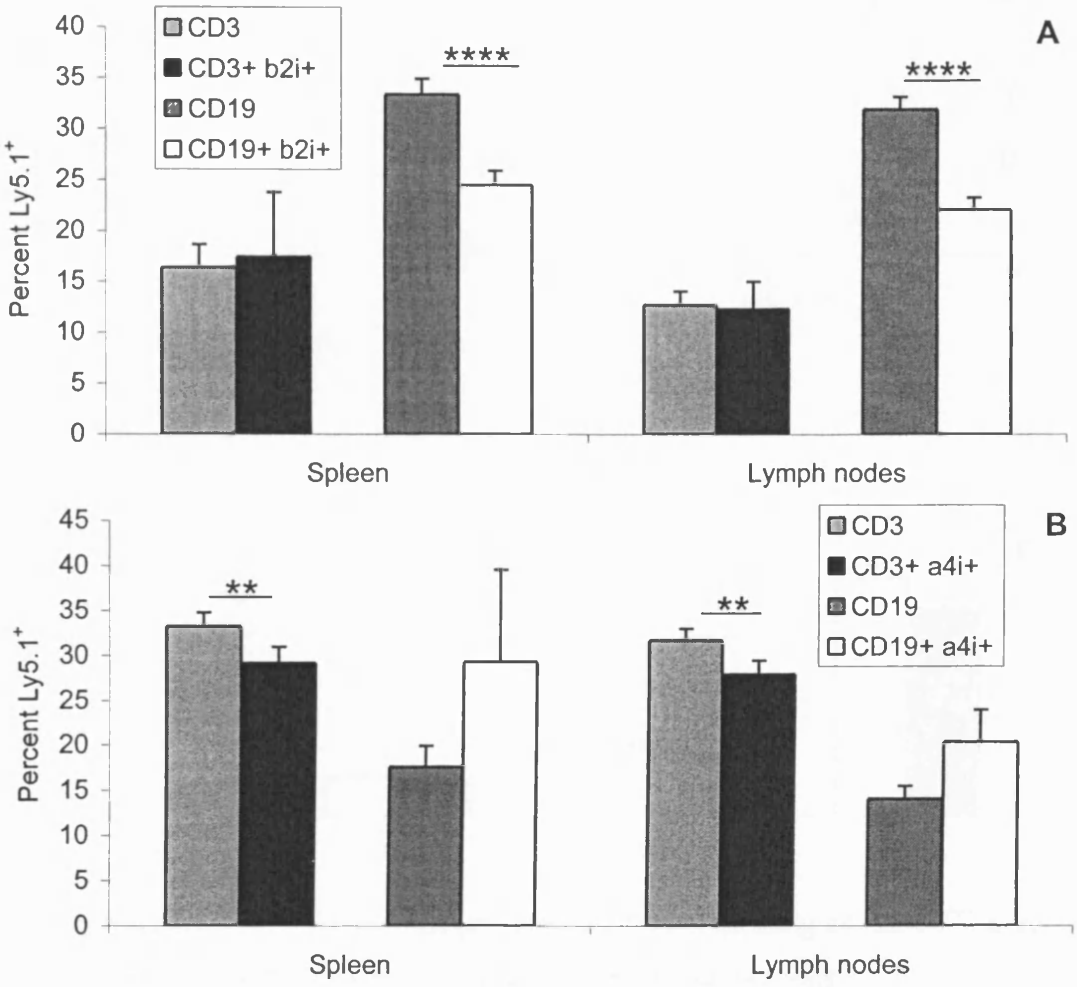

Figure 4.30. Analysis of cells positive for two integrins in HSC $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & HSC $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras

A. Percentage of $CD44^{-/-}$ ($Ly5.1^{+}$) cells found in total $CD3^{+}$ or $CD19^{+}$ populations compared to $CD3^{+}$ or $CD19^{+}$ $LPAM^{+}\beta_2integrin^{+}$ (b2i) cells. B. Percentage of $CD44^{-/-}$ ($Ly5.1^{+}$) cells found in $CD3^{+}$ or $CD19^{+}$ populations compared to $CD3^{+}$ or $CD19^{+}$ $LPAM^{+}\alpha_4integrin^{+}$ (a4i) cells. The data are mean ± s.e.m from groups of 4. P values, 2-sample t test; **, $P<0.01$, ****, $P<0.001$.

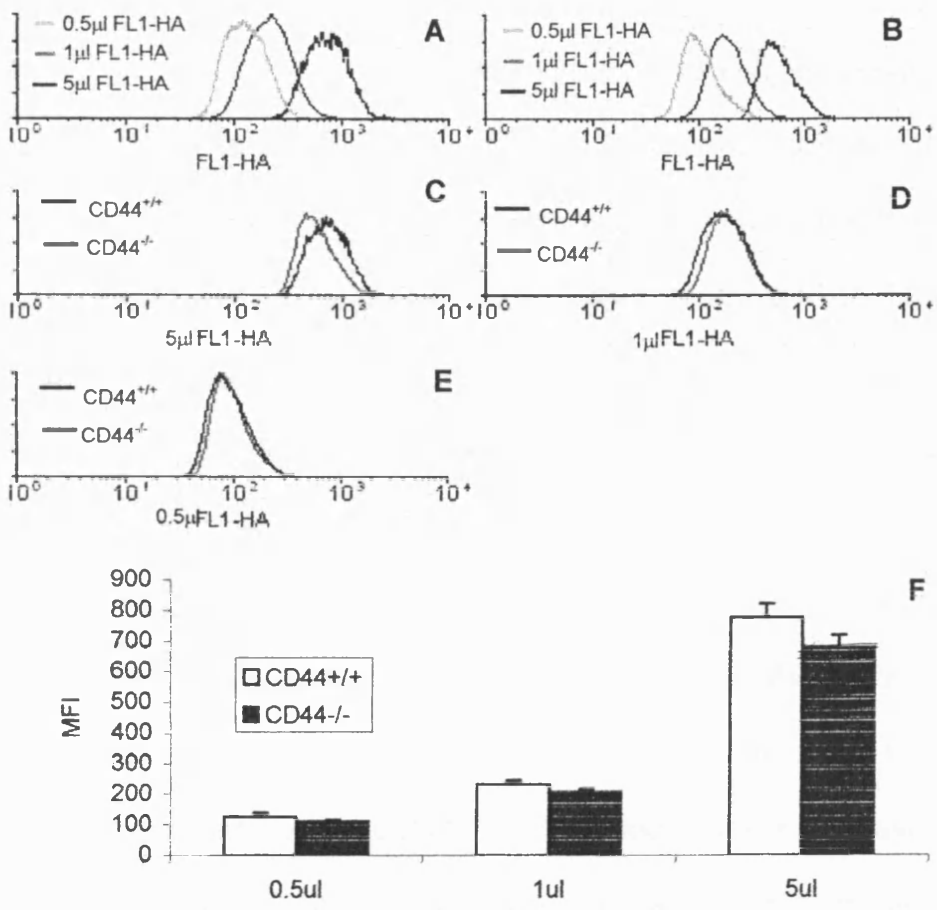

Figure 4. 31. FITC-Hyaluronate (FL1-HA) binding of $CD44^{+/+}$ and $CD44^{-/-}$ $CD11c^{+}$ *bone marrow derived dendritic cells.*

A. Titration of FL1-HA on $CD44^{+/+}$ derived bone marrow cells. B. Titration of FL1-HA on $CD44^{-/-}$ derived bone marrow cells. C-E Comparison of titration of FL1-HA on $CD44^{+/+}$ and $CD44^{-/-}$ bone marrow derived cells. C. 5µl FL1-HA per 10^6 cells. D. 1µl FL1-HA per 10^6 cells. E. 0.5µl FL-1HA per 10^6 cells. F. Summary FL-1-HA binding by $CD44^{+/+}$ and $CD44^{-/-}$ bone marrow derived cells (MFI). the data are mean ± s.e.m from groups of 3. A-E data representative of group of 3. Bone marrow derived cells obtained from age and sex matched $CD44^{+/+}$ or $CD44^{-/-}$ mice.

4.4.Summary.

To examine the role of CD44 in the homing of precursor cells to primary lymphoid organs, fluorescently labelled $CD44^{+/+}$ or $CD44^{-/-}$ lineage negative bone marrow cells were injected into mice. Contrary to prediction, there was no difference in the ability of the $CD44^{-/-}$ cells to enter the bone marrow compared to $CD44^{+/+}$ cells, whether they were co-injected or injected individually. No labelled cells were detected in the thymus after 48 hours, although this was not unexpected. As the majority of lineage positive cells were removed, it would take longer for the cell to develop into thymic progenitors and migrate to the thymus, in sufficient numbers so as to be detectable. There were however differences in the blood 12hrs post injection, where more $CD44^{-/-}$ cells were found after co-injection with $CD44^{+/+}$ cells. This could be explained by the $CD44^{+/+}$ cells being quicker to leave the blood stream and filling niches in either the bone marrow or other tissues, therefore leaving the $CD44^{-/-}$ cells in the circulation. Interestingly, there were more $CD44^{-/-}$ cells isolated from the blood when co-injected with $CD44^{+/+}$ cells than when injected alone, even though twice as many cells were initially injected in the latter situation. This could lend further support to the idea that the $CD44^{+/+}$ cells competed with $CD44^{-/-}$ for exit from the blood stream. Any differences between the division rates of $CD44^{+/+}$ and $CD44^{-/-}$ cells could not be deciphered due to unequal CFSE staining. However, it was apparent was that there was a higher division rate or a greater loss of CFSE staining in the cells that were injected singularly compared to those that were co-injected, although this was not apparent by 48hrs. The intra-thymic injections demonstrated that after 4 weeks there were approximately the same number of $CD44^{+/+}$ and $CD44^{-/-}$ T cells in the spleen, either

when the precursors were co-injected or when $CD44^{+/+}$ or $CD44^{-/-}$ cells were injected individually. Thus, intra-thymic injections appeared to abrogate the deficit shown by the $CD44^{-/-}$ precursors in producing T cells in the normal mixed chimeras. However, an unexpected finding was that donor B cells were generated in mice injected intra-thymically with precursors, indicating that injected cells were not restricted to the thymus. In addition, it is interesting to note that much lower numbers of B cells were produced from $CD44^{-/-}$ cells early after intra-thymic injection, irrespective of whether they were co-injected with $CD44^{+/+}$ cells or injected individually. Therefore it is possible that the number of $CD44^{+/+}$ and $CD44^{-/-}$ progenitors remaining in the thymus after intra-thymic injection was actually biased in favour of $CD44^{-/-}$ cells. If this were the case, the data would still be compatible with the idea that $CD44^{-/-}$ cells have a reduced ability to produce T cells. Notably, 6 weeks after intra-thymic injection, the phenotype again resembled the mixed chimeras with the $CD44^{-/-}$ cells producing fewer T cells and more B cells. The T cell subsets, again showed variation between the two time points. After 4 weeks, there were predominantly $CD8^+$ T cells, except in the $CD44^{+/+}$ only injected mice. After 6 weeks the normal $CD4^+$ T cell dominance was observed. At 4 weeks there was no difference in the contribution of $CD44^{+/+}$ or $CD44^{-/-}$ cells to the $CD4^+$ T cell population, but among $CD8^+$ T cells a significantly higher number were $CD44^{-/-}$ derived. After 6 weeks there was normal $CD44^{+/+}$ cells dominance in the $CD4^+$ and $CD8^+$ T cell populations, in the co-injected chimeras.

One explanation for these complicated findings is that they are linked to the timing of mature T cell migration from the thymus and the replenishment of the thymus with precursors from the bone marrow. For example $CD8^+$ T cells derived from the

intra-thymically injected cells may have matured and left the thymus in the co-injected and $CD44^{-/-}$ only injected mice increasing the percentage of CD8 T cells in the periphery, whilst the CD4+ cells derived from the intra-thymic injected cells are still in the thymus. Whilst in the $CD44^{+/+}$ injected mice, the majority of the injected cells had migrated to the bone marrow and subsequently seed the thymus with T cell precursors, at a normal rate. Conversely intra-thymic injection may allow for the predominant generation of $CD8^+$ T cells rather than $CD4^+$ T cells. The bias of $CD44^{+/+}$ $CD8^+$ T cells versus $CD44^{-/-}$ $CD8^+$ T cells only in the co-injected mice is compatible with the idea that $CD44^{-/-}$ cells remain in the thymus after intra-thymic injection, whilst the $CD44^{+/+}$ cells migrate easily out. However in the $CD44^{-/-}$ only mice, the $CD44^{-/-}$ cells fill the empty niches in the thymus and the excess migrate out with difficulty. After 6 weeks, in the co-injected mice there were again fewer $CD44^{-/-}$ T cells, among both $CD4^+$ and $CD8^+$ cells. In the individually injected mice, there were more $CD44^{-/-}$ than $CD44^{+/+}$ $CD8^+$ T cells.

There was little difference in the representation of $CD44^{-/-}$ cells among the $CD4^+$ T cell subsets after 4 weeks in the co-injected mice. In the individually injected mice, there were significantly more $CD4^+$ T cells generated from $CD44^{+/+}$ than $CD44^{-/-}$ cells. The ratio of $CD25^-CD4^+$:$CD25^+CD4^+$ T cells, was similar to that in the mixed chimeras in that the $CD44^{-/-}$ cells produced a higher frequency of $CD25^+$ $CD4^+$ T cells than the $CD44^{+/+}$ $CD4^+$ T cells, in both the co-injected and the individually injected mice.

Therefore, it appears as though the intra-thymic injection of precursors can not be used to fully address the reasons for the differences between $CD44^{+/+}$ and $CD44^{-/-}$ cells in T cell production, as there appears to be differences in their migrational

properties which make the reconstitution unequal. Nevertheless, it can be tentatively concluded that, partially of the defects exposed in the previous chapter can be explained by deficits in the ability of $CD44^{-/-}$ to enter the thymus. Although they appear to develop normally once they have gaining entry into the thymus, there was still a difference in $CD4^+$ vs. $CD8^+$ T cell and $CD25^-CD4^+$ vs. $CD25^+CD4^+$ T cell development, which seems to be inherent to the $CD44^{-/-}$ T cells.

When the ratios of $CD44^{+/+}$:$CD44^{-/-}$ cells was calculated and compared in different organs, it appeared that there could be a difference in the migratory properties of the $CD44^{+/+}$ and $CD44^{-/-}$ B and T cells, although the results were not wholly consistent. Mainly, it appeared as though there were proportionally more $CD44^{-/-}$ B cells in the LN than the spleen of most mice, regardless of the host environment. It was also found that the peritoneal cavity and the liver contained fewer $CD44^{-/-}$ B cells than most organs which was indicated in the previous chapter. Whether these differences are due to an impairment of the $CD44^{-/-}$ cells to enter the tissues, and/or remain there is not known.

The previous section exposed potential differences in migration of the $CD44^{+/+}$ and $CD44^{-/-}$ cells, and after examination of a panel of adhesion molecules in two groups of chimeras, it was found that there were differences between $CD44^{+/+}$ and $CD44^{-/-}$ T and B cells. Many differences were found, but the following were consistent between groups. It appeared that $CD44^{-/-}$ splenic B cells had an increased expression of CD62L, but a decreased expression of CD48 and LPAM1. In the LN, $CD44^{-/-}$ B cells had decreased in CD48, LPAM1 and CD103 expression, but increased expression of CD11b, $\beta_2$integrin, and $CD48^{hi}$. $CD44^{-/-}$ splenic T cells mainly had an increase in their expression of adhesion molecules, particularly

CD11b and CD102, whilst in the LN $CD44^{-/-}$ T cells had an increased CD11b, CD11a and LPAM1 expression.

It was surprising to note that DCs derived from $CD44^{-/-}$ mice could also bind hyaluronate. This is in contradiction to a report by Stoop *et al*, where they reported that neither $CD44^{+/+}$ nor $CD44^{-/-}$ DCs could bind it. One difference could be the different background (DBA/2) of the mice used in the previous study. The lack of a negative control in this experiment could provide some doubt about the specificity of the FL1-HA. Therefore this experiment should be repeated using an anti-CD44 antibody which has been demonstrated to block CD44-HA interactions. However other molecules could bind CD44 such as RHAMM. The lack of cell surface expression of CD44 on the $CD44^{-/-}$ mice had previously been confirmed by FACS analysis.

In conclusion, there do appear to be subtle defects in the ability of $CD44^{-/-}$ cells to enter both primary and secondary lymphoid tissues, though not the bone marrow. Nevertheless, it was also interesting to note from the intra-thymic injections that $CD44^{+/+}$ but not $CD44^{-/-}$ cells appeared to migrate out of the thymus and into the bone marrow, even though they were co-injected.

Results in the previous chapter suggested that $CD44^{-/-}$ cells had a deficit in differentiating into T cells and certain T and B cell subsets. These differences in T cell subset differentiation were not eliminated by intra-thymic injections. This chapter has also shown that the $CD44^{-/-}$ cells also have a slight impairment in entering into tertiary tissues. In addition, when injected into the adult thymus $CD44^{-/-}$ progenitors still display deficits in generating T cells. Therefore, reduced

generation of T cells is not solely a problem of homing of precursors to the thymus. Similarly, differences in B cell generation do not appear to be due to alterations in homing to the bone marrow, as the $CD44^{+/+}$ and $CD44^{-/-}$ cells homed equally to the bone marrow. These data suggest, therefore CD44 may be involved in directing lymphocyte differentiation at some stage.

These chimeras were also generated using a higher dose of irradiation and potential stromal and micro-endothelial damage could occur, which could alter the migratory abilities of the B and T cells.

As these were analysed using anti-Ly9.1 antibodies, T cell ratios were calculated eliminating host T cells, and the standard 900rads irradiation was used, thereby lessening the stromal and vascular damage.

5. Chapter 5: CD44 involvement in T and B cell development.

5.1. Introduction

T and B cell development in the adult begins in the BM where a pluirpotent self-renewing HSC is thought to develop using, the PU-1 transcription factor, into a common myeloid and lymphoid progenitor (244). Uptake of HSCs into the BM is thought to involved CD44 as both a receptor for HA and a ligand for E-selectin (155, 245). This common myeloid lymphoid progenitor then develops into a common lymphoid progenitor by the action of the Ikaros transcription factor. This common lymphoid progenitor can then differentiate into the T, B, NK or DC development pathway.

5.1.1. B cell development

B cells develop from common lymphoid progenitors derived from pluripotent haematopoietic stem cells, with the action of E2A, early B cell factor (EBF) and PAX5, transcription factors (246). Their development can be divided into four stages, based on the work by Hardy. The first stage, consists of pro-B cells which are $CD43^{+}B220^{lo}sIgM^{-}$, and represent 5% of the BM cells. The adhesive interaction between the pro-B cells and the BM stroma, essential for B cell development is mediated by CD44, and its variant isoforms (224-226, 247). This early pro-B cell stage can be divided into many different stages based on gene

rearrangement, enzymes present (e.g. TdT), and the responsiveness of cells to cytokines (e.g. IL-7). At the beginning of this stage, cells can differentiate into other lineages. However, by the end of this stage, the heavy chain genes have been rearranged and cells are committed to the B cell development pathway. In addition, many cells have been lost to apoptosis due to unsuccessful gene rearrangements. It is in this stage that the $RAG^{-/-}$ deficiency leads to the apoptosis of all $RAG^{-/-}$ B cells for they can not create the double stranded DNA breaks needed for the Ig-H chain gene rearrangements.

The next stage consists of pre-B cells, which are $CD43^{-}sIgM^{-}B220^{lo}$, which represent 30% of BM cells. These cells have cytoplasmic μH chain expression, are rearranging their light chain genes, and express a pre-B cell receptor on the cell surface. They undergo negative selection at this stage and the majority of cells are deleted via apoptosis, although it has been found that immature B cells recognising an autogen, can also rearrange their light chain instead of being deleted.

The last developmental stage in the BM is that of immature B cells, which are $CD43^{-}B220^{lo}sIgM^{+}$. These cells have undergone both Ig-L and Ig-H chain rearrangement, and express IgM on the cell surface. They undergo rapid proliferation, producing approximately 20% of the BM cells. At this stage, B cells leave the BM to undergo the next stage of B cell development in the spleen. The majority of B cells are thought to die after entering the circulation, while a minor population undergoes positive selection in the spleen, before re-joining the circulation.

Finally, mature re-circulating B cells are also found in the bone marrow. These cells are $CD43^{-}IgM^{+}B220^{hi}sIgD^{+}$ and make up 5% of BM cells.

5.1.2. T cell development

T cell progenitors are thought to develop from the common lymphoid progenitor in response to notch1 signalling, which is activated by association of notch1 with its ligands present on the stroma. Whether the activation of the common lymphoid progenitor to develop into a pre-T cell by notch signalling occurs in the thymus or in the BM is unclear. Interestingly, though in the absence of notch signalling, lymphoid progenitors develop into B cells in the thymus (248).

T cell progenitors exported from the BM via the blood, enter the thymus through the venules into the medulla. The initial interactions between the pre-T cell and the thymic stroma has been shown to be blocked by CD44- and variant CD44-specific antibodies (128). Entry of the thymocytes into the double negative (DN, $CD4^{-}$, $CD8^{-}$) stage of T cell development is thought to occur in the sub-capsular zone of the thymus and takes approximately two weeks to complete. The DN stage of T cell development can be further split into four stages based on CD44 and CD25 expression. In first stage, or DN1 ($CD44^{+}CD25^{-}$), the cells are still pluirpotent they can give rise to B cells, NK cells and DCs, as well as T cells, and they also have a low division rate. In second stage, or DN2 ($CD44^{+}CD25^{+}$), the cells are not as pluripotent as in the previous stage as they can only give rise to DCs apart from T cells, and the cells have a very high division rate due to influences of the cytokines

c-kit and IL-7. Survival of this subset is thought to also be dependent upon notch signalling (249). The cells in the third stage or DN3 ($CD44^-CD25^+$), is thought only to be capable of producing immature T cells; these resting immature T cells begin β, γ and δ TCR germline rearrangement, and the γδ T cells are thought to be generated. It is at this stage in T cell development, when $RAG^{-/-}$ host T cells undergo apoptosis, due to an inability to generate the double stranded DNA breaks needed for the rearrangement of the TCR germline. Cells in the fourth stage, or DN4 ($CD44^-CD25^-$), again have a high turnover. TCR β germline rearrangement has finished, and γ δ rearrangement will be completed at the end of this stage. In addition, the pre-TCR is expressed at the cell surface and TCRα germline rearrangement is being initiated. Adhesive interactions between the thymocytes and the thymic epithelium and mesenchymal fibroblasts allow the DN2 cells to progress to DN3, while the thymic epithelium alone is necessary for the DN3 to develop into the double positive stage (250).

CD4 or CD8 molecules are individually expressed on the surface of the DN thymocytes to generate a brief immature single positive stage (ISP). The significance of the ISP stage is not understood but this phenotype may be due to differential kinetics of the CD4 and CD8 molecules being expressed at the cell surface.

The immature cells subsequently enter the double positive (DP) stage of T cell development ($CD4^+$ $CD8^+$), which typically lasts for about four days; these cells are found in the cortex of the thymus. During this stage, TCR α germline rearrangement takes place, and the TCR is expressed on the cell surface. There is little cell division occurring in this stage, and due to positive and negative

selection, up to 95% of the cells will apoptose. A more detailed account of thymocyte selection can be found in the introduction.

Cells interacting productively with either MHCI or MHCII will then continue into the next stage of T cell development. Successful interaction with MHCI will result in the cell proceeding to the CD8 single positive stage, and successful interaction with MHCII will result in the cell proceeding to the CD4 single positive stage. Although the precise mechanisms involved are not fully understood, there is evidence that notch1 is involved in the CD8 vs. CD4 T cell lineage decision. It appears as though a stronger notch1 signal leads to $CD8^+$ T cells whereas $CD4^+$ T cells develop with a weaker notch signal (33, 251-253).

The final stage of thymocyte maturation is the single positive stage, which takes approximately two weeks to complete. During this stage thymocytes undergo the last processes of selection in the medulla and up-regulates CD3. The mature T cells leave the thymus through an active process via the blood and the lymph vessels at the corticomedullary junction. The precise mechanism by which this exit occurs is unknown.

Results in chapter 3 showed differences in T and B cell development of $CD44^{+/+}$ and $CD44^{-/-}$ cells when placed in competition with each other. Deficiencies that $CD44^{-/-}$ cells may have had in generating T cells were not eliminated by intra-thymic injection, and the differences observed in B cell generation were not due to altered BM homing. These findings suggest that CD44 may have a role in regulating the differentiation of lymphocytes within the primary lymphoid organs.

5.2. Aim

The aim of this chapter is to investigate to possibility that CD44 is involved in T cell and/or B cell development. We will determine whether CD44 is involved in T cell development by examining T cell development in the thymus from donor $CD44^{+/+}$ and $CD44^{-/-}$ progenitors. We will also determine whether CD44 is involved in B cell development by comparing donor $CD44^{+/+}$ and $CD44^{-/-}$ B cells at different stages of development.

5.3.Results: A role for CD44 in T cell development

CD44 has been demonstrated to facilitate T cells in entering the thymus. In the previous chapters, $CD44^{-/-}$ progenitors had a reduced ability to produce mature T cells in chimeras made in either $CD44^{-/-}$ or $CD44^{+/+}$ hosts. This was only apparent with the presence of competing $CD44^{+/+}$ progenitors, since the $CD44^{-/-}$ progenitors can fully fulfil the T cell quota without the presence of competing $CD44^{+/+}$ progenitors. It has also been indicated, that $CD44^{-/-}$ T and B cells appear to have differences in their adhesion molecule profiles, possibly to enable them to enter tissues in the absence of CD44. These pieces of information lend support to the idea that CD44 may be more involved in T cell development in the thymus than just in thymic entry. Therefore the proportion of $CD44^{-/-}$ at different stages of T cell development were examined in several types of chimeras.

5.3.1.1. $CD44^{+/+}$ ($Ly5.2^+$) and $CD44^{-/-}$ ($Ly5.1^+$) donors → $CD44^{+/+}$ ($Ly5.2^+$) chimeras.

Chimeras were generated as described in section 3.3.1. The percentage and distribution of total, $Ly5.1^+$ and total $Ly5.1^-$ cells were analysed in the thymus of the chimeras. There was a significantly higher proportion of $Ly5.1^+$ ($CD44^{-/-}$) cells than $Ly5.2^+$ ($CD44^{+/+}$) cells found among both the DN and the CD8SP populations in the thymus of these chimeras (Figure 5.1A). This was reinforced when the percentage of $Ly5.1^+$ cells found in each thymic subset was analysed. 12% of the double negative cells were $Ly5.1^+$, which was statistically higher than the other subsets, for which the percentage ranged between 5 and 8%. CD8SP also had a statistically higher percentage of $Ly5.1^+$ cells than both CD4SP and DP populations (Figure 5.1B). As an approach to assess whether the rate of progression between developmental stages may differ between $CD44^{-/-}$ and $CD44^{+/+}$ thymocytes the proportion of $Ly5.1^+$, $Ly5.1^-$ or total cells present in a particular population was divided by the proportion present in the subsequent population (Figure 5.1C). Differences were observed between the $Ly5.1^+$ and $Ly5.1^-$ populations in the ratio of DN to DP cells, where $Ly5.1^+$ cells had a higher ratio than $Ly5.1^-$ cells. Conversely, $Ly5.1^+$ cells had a lower ratio of DP to CD8SP cells.

5.3.1.2. $CD44^{+/+}$ ($Ly5.2^+$) and $CD44^{-/-}$ ($Ly5.1^+$) donors → $CD44^{-/-}$ ($Ly5.1^+$) chimeras.

To determine whether CD44 expression by the host influenced the development of $CD44^{-/-}$ and $CD44^{+/+}$ thymocytes, chimeras were generated in $CD44^{-/-}$ recipients as described in section 3.3.2. In these mice, there was a significantly higher

proportion of Ly5.1$^+$ (CD44$^{-/-}$) cells than Ly5.2$^+$ (CD44$^{+/+}$) cells in the double negative population, and a significantly lower proportion of Ly5.1$^+$ cells in the double positive subset (Figure 5.2A).

Strikingly, 30% of the DN cells were Ly5.1$^+$, whereas the percentage of Ly5.1+ cells in the double positive stage was only 2% (Figure 5.2B). There was also a statistically lower percentage of Ly5.1$^+$ cells among DP cells than in both consecutive stages, the CD4SP and CD8SP subsets. When the ratio of cells in progressive stages of differentiation was determined, there were differences between the Ly5.1$^+$ and Ly5.1$^-$ populations in both the DP to CD4SP, the DP to CD8SP stages where on both occasions, Ly5.1$^+$ cells had a lower ratio than the Ly5.1$^-$ cells (Figure 5.2C). Although, there was again a higher DN to DP ratio among Ly5.1$^+$ cells.

5.3.2. CD229 expression in T cell development

CD229 (Ly9) was utilised in experiments described in the previous chapter to help to elucidate the role of CD44 in T cell development. To ensure that it was of suitable use as a marker in T cell development, its expression was compared with that of CD2 on thymocyte subsets. Ly9.1 was found on thymocytes at all stages of thymic development in 129Sv mice but (as expected) was not expressed in B6 mice (Figures 5.3 & 5.4). In contrast, CD2 was expressed only on a proportion of DN and DP thymocytes, but on virtually all CD4SP and CD8SP cells. This finding was consistent in both strains of mice.

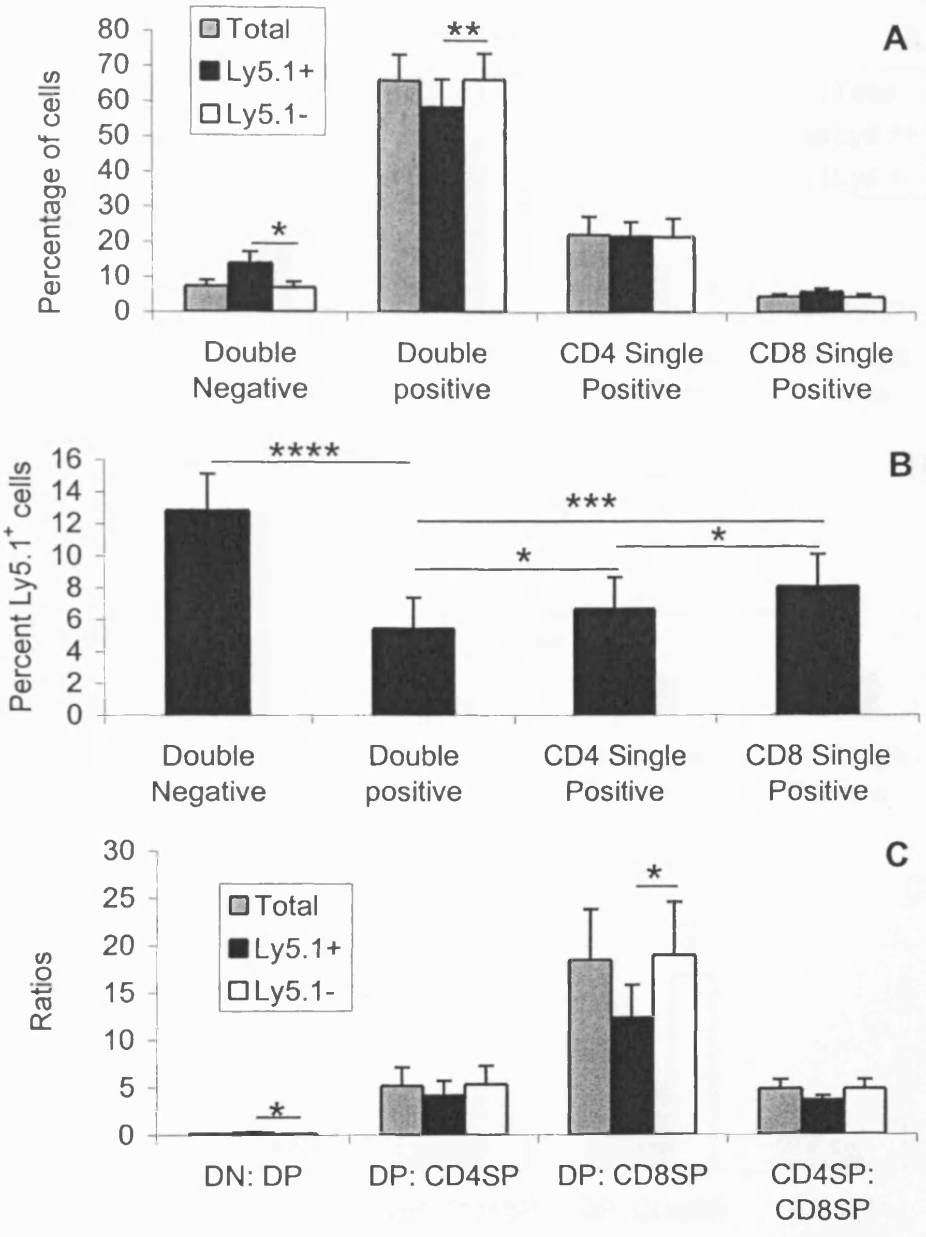

Figure 5.1. Analysis of thymic subsets in CD44$^{+/+}$ (Ly5.2^{+}) & CD44$^{-/-}$ (Ly5.1^{+}) → CD44$^{+/+}$ (Ly5.2^{+}) chimeras.

A. Distribution of total, Ly5.1^{+} and Ly5.1^{-} cells in different thymic subsets. B. Percentage of Ly5.1^{+} (CD44$^{-/-}$) cells in different thymic subsets. C. Ratio of total, Ly5.1^{+} and Ly5.1^{-} cells in progressive T cell developmental stages. The data are mean ± s.e.m. from 2 independently made chimeric groups of 3. P values, 2-sample t test; *, P<0.05, ***, P<0.005, ****, P<0.001.

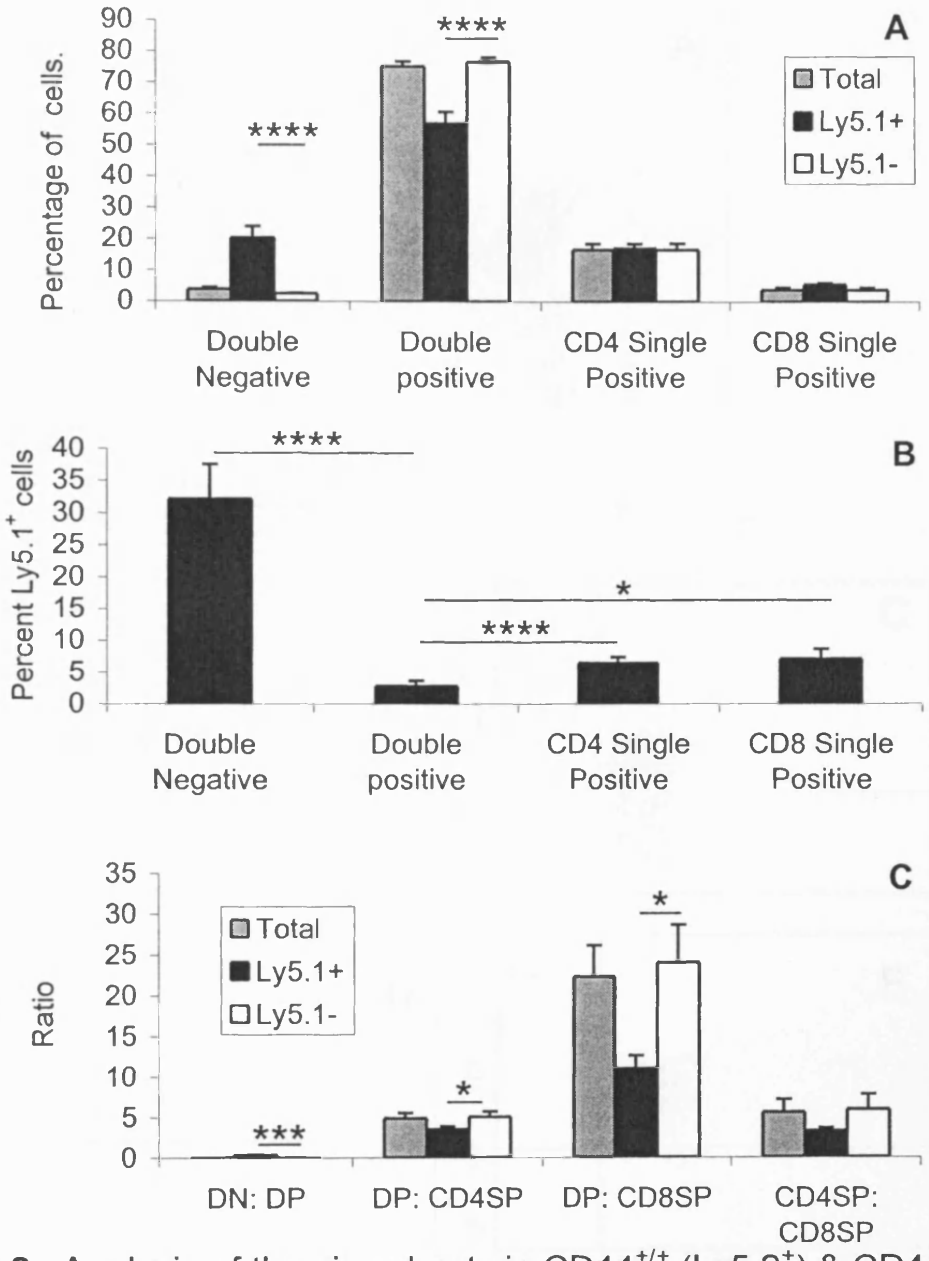

Figure 5.2. Analysis of thymic subsets in $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly5.1^{+}$) → $CD44^{-/-}$ ($Ly5.1^{+}$) chimeras.

A. Distribution of total, total $Ly5.1^{+}$ and $Ly5.1^{-}$ cells in different thymic subsets. B. Percentage contribution of $Ly5.1^{+}$ ($CD44^{-/-}$) in different thymic subsets. C. Ratio of distribution of total, total $Ly5.1^{+}$ and $Ly5.1^{-}$ cells in progressive T cell developmental stages. The data are mean ± s.e.m. from 2 chimeric groups of 4. P values, 2-sample t test; *, $P<0.05$, ***, $P<0.005$, ****, $P<0.001$.

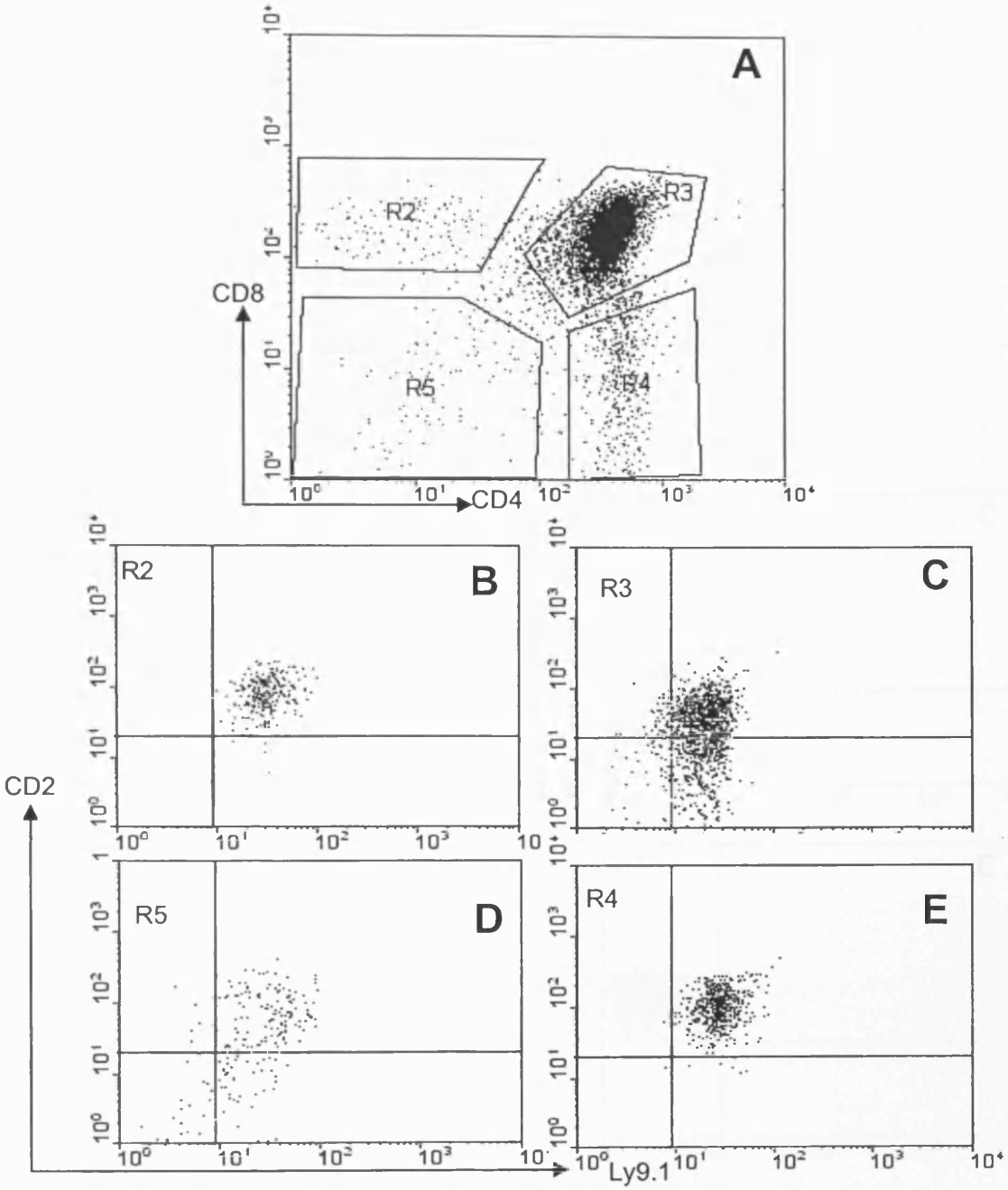

Figure 5.3. Distribution of Ly9.1 and CD2 on various thymic subsets in 129/Sv mice

A. CD4 and CD8 expression on thymocytes. Polygons show gates used for defining 4 cell subsets. B. CD2 vs. Ly9.1 expression on CD8 single positive cells. C. CD2 vs. Ly9.1 expression on double positive cells. D. CD2 vs. Ly9.1 expression on double negative thymocytes. E. CD2 vs. Ly9.1 expression on CD4 single positive cells. Data are representative of results from 2 129/Sv mice.

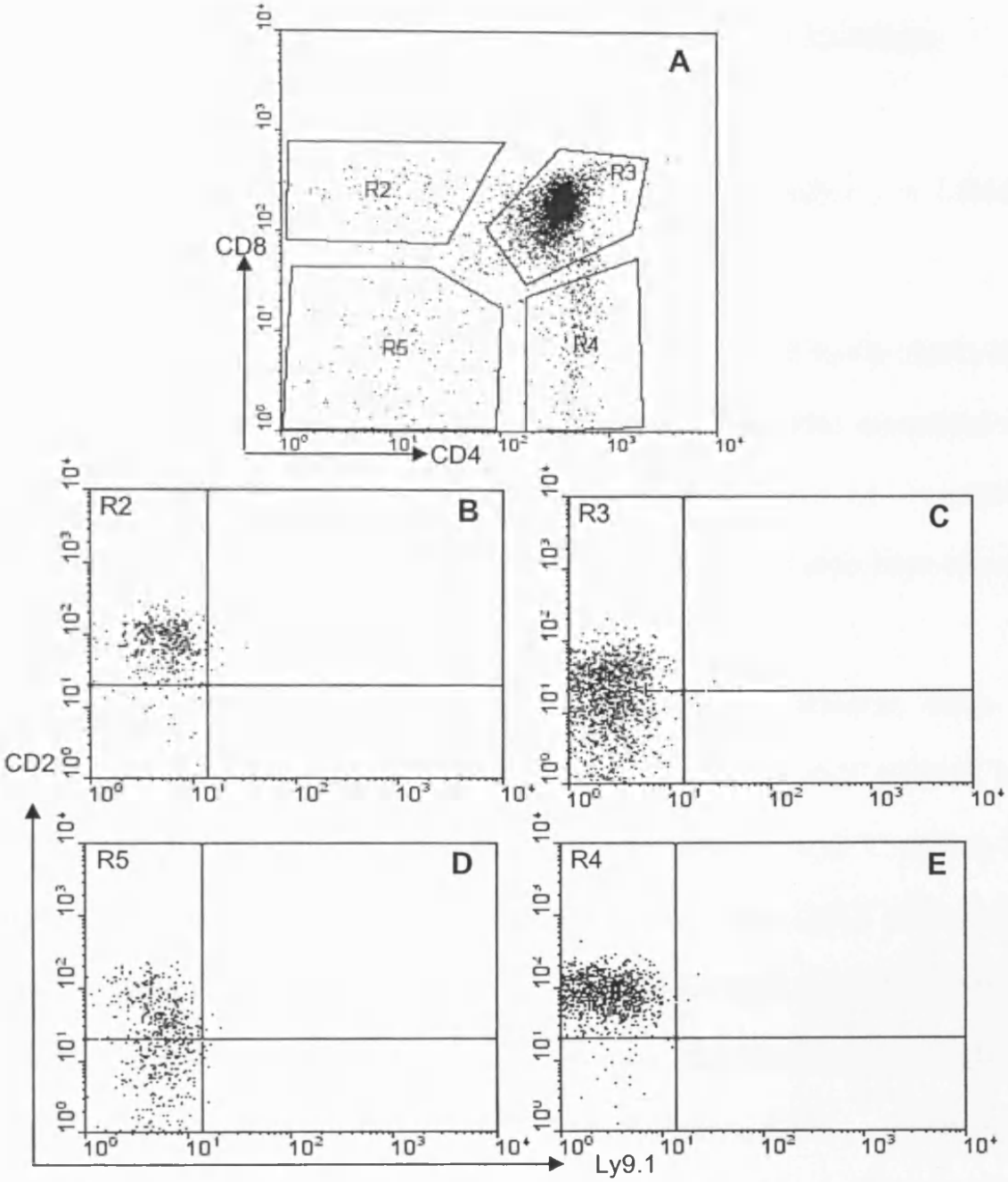

Figure 5.4. Distribution of Ly9.1 and CD2 on various thymic subsets in C57/Bl6 mice.

A. CD4 and CD8 expression on thymocytes. Polygons show gates used for defining four cell subsets. B. CD2 vs. Ly9.1 expression on CD8SP cells. C. CD2 vs. Ly9.1 expression on DP cells. D. CD2 vs. Ly9.1 expression on DN thymocytes. E. CD2 vs. Ly9.1 expression on CD4SP cells. Data are representative of results from 2 C57/Bl6 mice.

5.3.3. T cell development in secondary mixed chimeras

5.3.3.1. $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) and $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.1^+$) 1100 chimeras

To reduce the possible contributions of radioresistant T cells to the distribution and composition of donor cells in the thymus, Chimeras were also generated using a higher dose of irradiation (1100 rads) as described in section 3.3.6. In addition, the Ly9 marker was used together with Ly5 to distinguish donor cells from any residual host T cells.

As in mixed chimeras made using a lower dose of radiation, there was a significantly higher proportion of $Ly9.1^+$ ($CD44^{-/-}$) cells than $Ly5.2^+$ ($CD44^{+/+}$) cells in the DN population, and a significantly lower proportion of $Ly5.1^+$ cells in the DP subset. There was also a higher proportion of $Ly9.1^+$ than $Ly5.2^+$ cells in both SP thymic subsets (Figure 5.5A). While 4% of the DN donor cells were $Ly9.1^+$, only 0.5% of DP donor cells were $Ly9.1^+$, in addition, significantly fewer $Ly9.1^+$ cells were DP than either CD4SP or CD8SP subsets (Figure 5.5B). When the ratios between progressive differentiation stages were determined, significant differences were again observed in the DN:DP ratio, where, as previously, $Ly9.1^+$ cells had a higher ratio than $Ly5.2^+$ cells. There were also differences between the $Ly9.1^+$ and $Ly5.2^+$ populations in both the DP:CD4SP, and the DP:CD4SP ratios, where in both cases, $Ly9.1^+$ cells had a lower ratio than the $Ly5.2^+$ cells. This again is in agreement with the previous chimeras (Figure 5.5C).

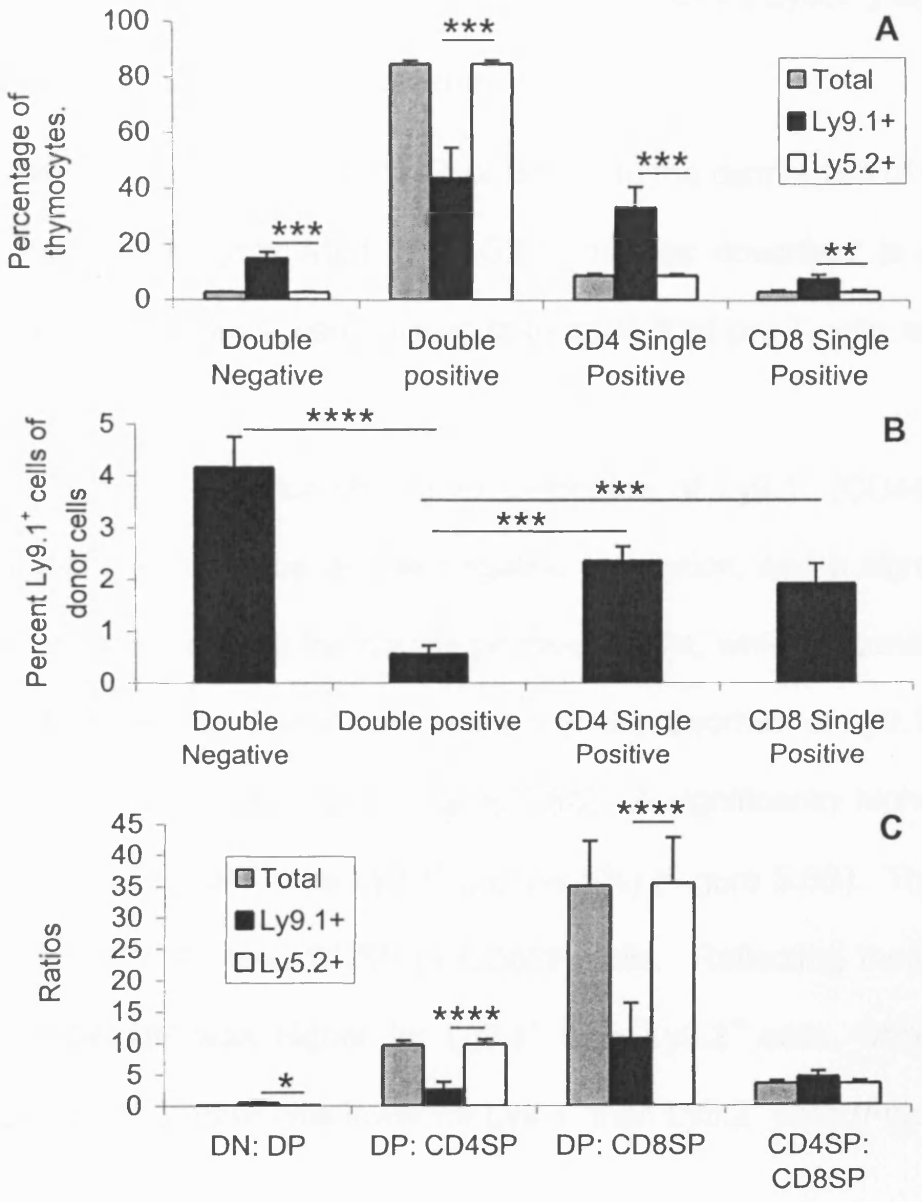

Figure 5.5. Analysis of thymic subsets in $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) and $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.1^{+}$) 1100 rad chimeras.

A. Distribution of total, $Ly9.1^{+}$ and $Ly5.2^{+}$ cells in different thymic subsets. B. Percentage of $Ly9.1^{+}$ ($CD44^{-/-}$) of donor cells in different thymic subsets. C. Ratio of of total, $Ly9.1^{+}$ and $Ly5.2^{+}$ cells in progressive T cell developmental stages. The data are mean ± s.e.m. from 2 chimeric groups of 3. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$.

5.3.3.2. $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) and $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras

To eliminate any contribution of host T or B cells to the distribution of donor derived cells, chimeras were generated in $RAG2^{-/-}$ hosts as described in section 3.3.7. Both Ly9 and Ly5 markers were utilised to exclude host pre-T cells, which could be generated.

There was again a significantly higher proportion of $Ly9.1^+$ ($CD44^{-/-}$) cells than $Ly5.2^+$ ($CD44^{+/+}$) cells in the double negative population, and a significantly lower proportion of $Ly5.1^+$ cells in the double positive subset, which is consistent with the other mixed chimeras. There was also a higher proportion of $Ly9.1^+$ than $Ly5.2^+$ cells in the CD4SP thymic subset (Figure 5.6A). A significantly higher percentage of DN cells than DP cells were $Ly9.1^+$ (25%vs 6%) (Figure 5.6B). There were also fewer $Ly9.1^+$ DP cells than CDSP or CD8SP cells. Reflecting these differences, the ratio of DN:DP was higher for $Ly9.1^+$ than $Ly5.2^+$ cells, while the ratio of DP:CD4SP or DP:CD8SP was lower for $Ly9.1^+$ than $Ly5.2^+$ cells (Figure 5.6C).

5.3.3.3. HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras.

To eliminate the possibility that differing numbers of pre-T cells in the donor BM could alter the seeding balance of $CD44^{-/-}$ cells to $CD44^{+/+}$ cells in mixed chimeras, chimeras were generated using lineage depleted BM as described in section 3.3.9.

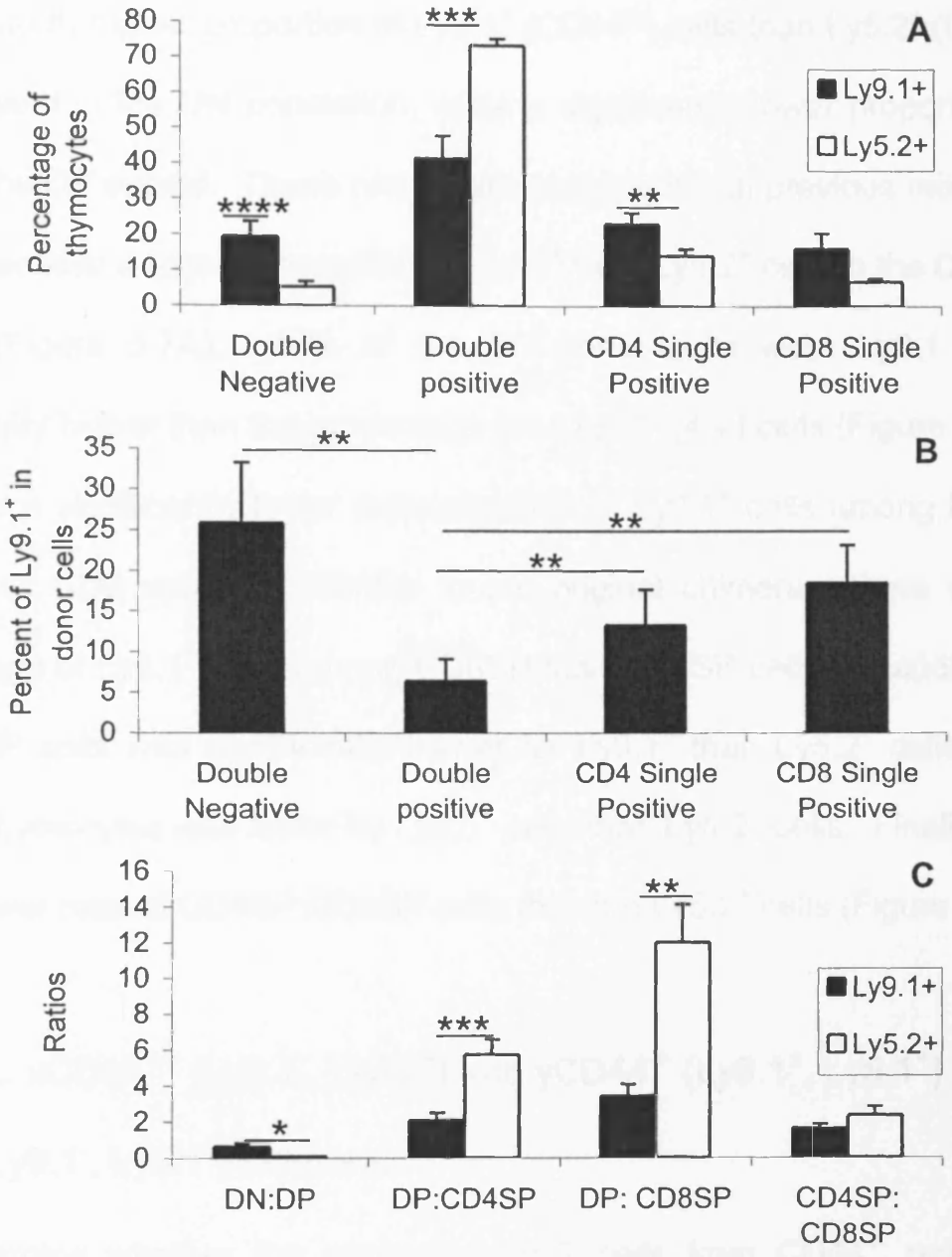

Figure 5.6. Analysis of thymic subsets in CD44$^{+/+}$ (Ly9.1^{-}, Ly5.2^{+}) and CD44$^{-/-}$ (Ly9.1^{+}, Ly5.1^{+}) → RAG2$^{-/-}$ (CD44$^{+/+}$, Ly9.1^{-}, Ly5.1^{+}) chimeras

A. Distribution of total, Ly9.1^{+} and Ly5.2^{+} cells in different thymic subsets. B. Percentage contribution of Ly9.1^{+} (CD44$^{-/-}$) in different thymic subsets. C. Ratio of Ly5.2^{+} and Ly9.1^{+} cells in progressive T cell developmental stages. The data are mean ± s.e.m. from 2 chimeric groups of 3. P values, 2-sample t test; *, P<0.05, **, P<0.01, ***, P<0.005, ****, P<0.001.

A significantly higher proportion of Ly9.1$^+$ (CD44$^{-/-}$) cells than Ly5.2$^+$ (CD44$^{+/+}$) cells was present in the DN population, while a significantly lower proportion of Ly5.1$^+$ cells in the DP subset. These results are consistent with previous mixed chimeras. There was also a higher proportion of Ly9.1$^+$ than Ly5.2$^+$ cells in the CD4SP thymic subset (Figure 5.7A). 15% of the DN donor cells were Ly9.1$^+$, which was significantly higher than the percentage among DP (4%) cells (Figure 5.7B). There was also a significantly lower representation of Ly9.1$^+$ cells among DP cells than CD4SP or CD8 subsets. Similar to the original chimeras, there was a higher percentage of Ly9.1$^+$ cells among CD8SP than CD4SP cells. In addition, the ratio of DN:DP cells was significantly higher for Ly9.1$^+$ than Ly5.2$^+$ cells, the ratio of DP:SP thymocytes was lower for Ly9.1$^+$ cells than Ly5.2$^+$ cells. Finally Ly9.1$^+$ cells had a lower ratio of CD4SP:CD8SP cells than the Ly5.2$^+$ cells (Figure 5.7C).

5.3.3.4. xCD44$^{+/+}$ (Ly9.1$^-$, Ly5.2$^+$) and yCD44$^{-/-}$ (Ly9.1$^+$, Ly5.1$^+$) → CD44$^{+/+}$ (Ly9.1$^-$, Ly5.1$^+$) chimeras.

To determine whether the production of T cells from CD44$^{-/-}$ progenitors was influenced by their relative frequency compared to CD44$^{+/+}$ cells, chimeras were generated using different ratios of CD44$^{-/-}$ and CD44$^{+/+}$ BM as described in section 3.3.8. Ly9 and Ly5 antibodies were used to distinguish donor and recipient cells.

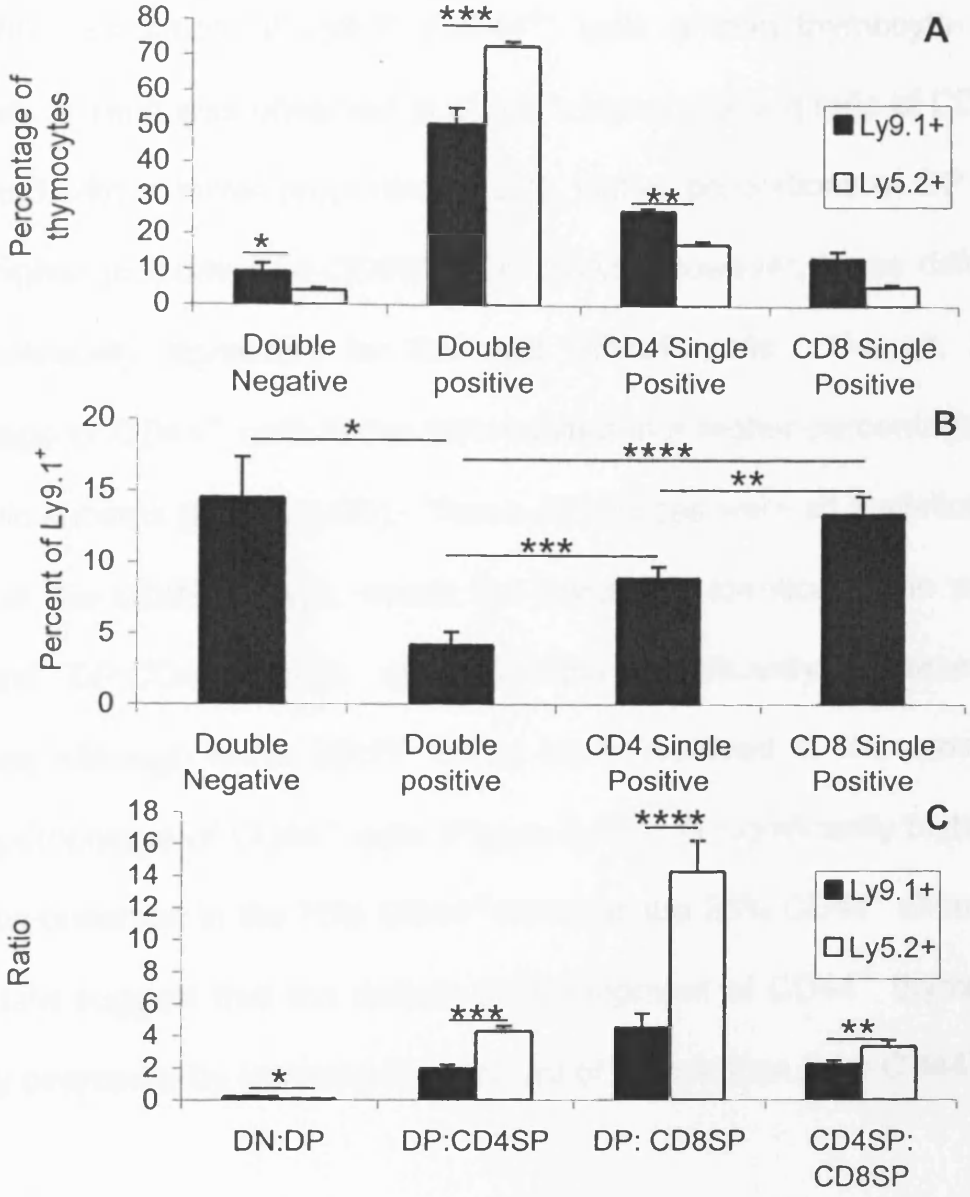

Figure 5.7. Analysis of thymic subsets in HSC CD44$^{+/+}$ (Ly9.1^{-}, Ly5.2^{+}) & HSC CD44$^{-/-}$ (Ly9.1^{+}, Ly5.1^{+}) → RAG$^{-/-}$ (CD44$^{+/+}$, Ly9.1^{-}, Ly5.1^{+}) chimeras.

A. Distribution of Ly9.1^{+} and Ly5.2^{+} cells in different thymic subsets. B. Percentage of Ly9.1^{+} (CD44$^{-/-}$) in different thymic subsets. C. Ratio of of Ly5.2^{+} and Ly9.1^{+} cells in progressive T cell developmental stages. The data are mean ± s.e.m. from 2 chimeric groups of 3. P values, 2-sample t test; *, P<0.05, **, P<0.01, ***, P<0.005, ****, P<0.001.

When the distribution of Ly9.1^{+} (CD44$^{-/-}$) cells among thymocyte subsets was examined, a trend was observed in which a higher starting ratio of CD44$^{-/-}$ BM was associated with a lower proportion of DN, higher proportions of DP and CD4SP, and a higher proportion of CD8SP (Fig 5.8A). However, these differences were only statistically significant for DN and CD8SP cells. Overall, an increased percentage of CD44$^{-/-}$ cells in the BM resulted in a higher percentage of CD44$^{-/-}$ in all thymic subsets (Figure 5.8B). These differences were all statistically significant except at the CD8SP stage, where the trend was identical. The ratio of DN:DP cells and DP:CD4SP cells, did not differ significantly between the various chimeras, although lower DN:DP ratios were observed in chimeras made with a higher percentage of CD44$^{-/-}$ cells (Figure 5.8C). A significantly higher DP:CD8SP ratio was observed in the 75% CD44$^{-/-}$ BM than the 25% CD44$^{-/-}$ chimeras. Overall, these data suggest that the defective development of CD44$^{-/-}$ thymocytes can be partially overcome by reducing the amount of competition from CD44$^{+/+}$ cells.

5.3.4. Investigation of the double negative (CD4^{-} CD8^{-}) thymic subset in various chimeras.

The previous section indicated that there were differences in T cell development found in the thymus of the mixed BM chimeras, which were irrespective of host. The mixed chimeras examined all consistently showed an increase in the percentage of CD44$^{-/-}$ cells present in the DN stage of thymic development, followed by a consistently low percentage of CD44$^{-/-}$ cells in the DP stage.

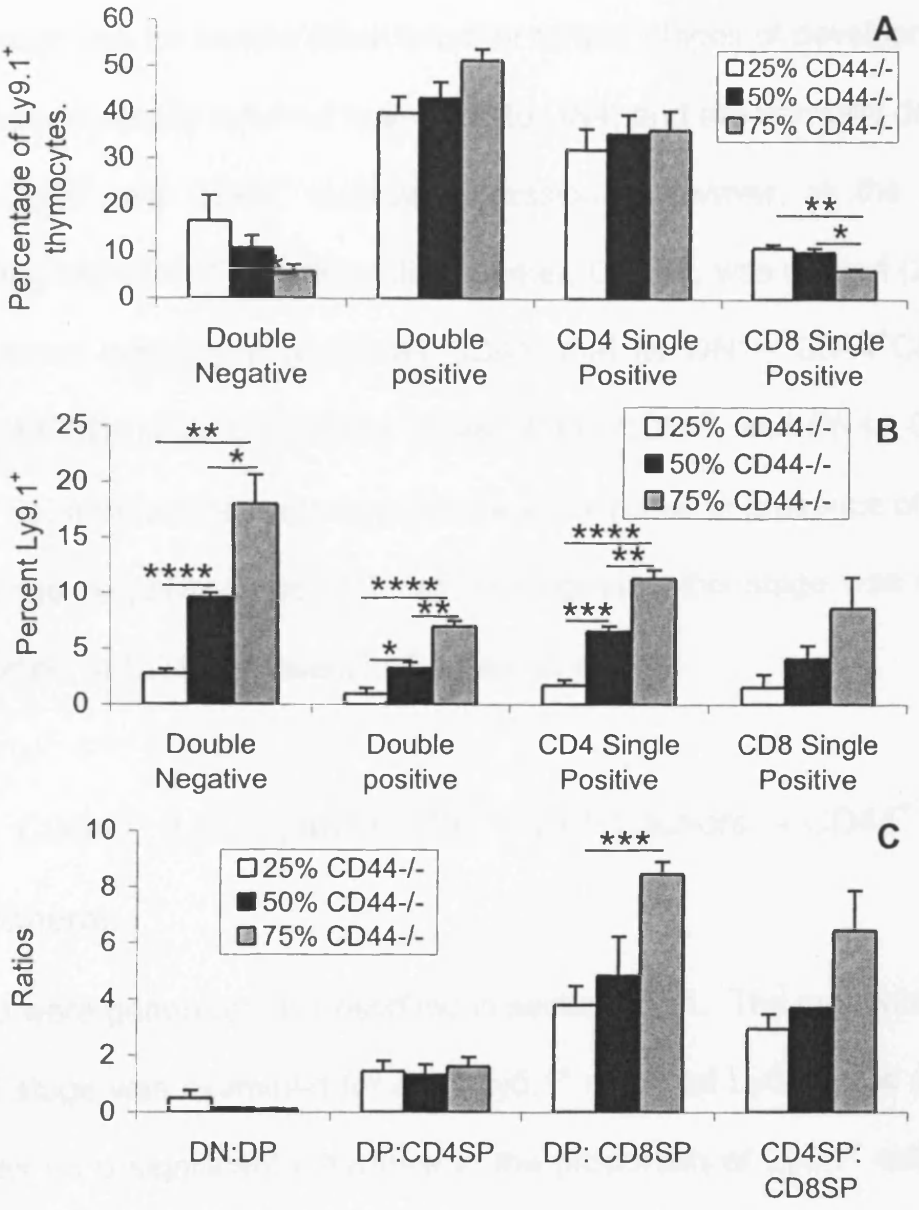

Figure 5.8. Analysis of thymic subsets in xCD44$^{+/+}$ & yCD44$^{-/-}$→ CD44$^{+/+}$ chimeras.

A. Distribution of Ly9.1^{+} cells in different thymic subsets of various chimeras. B. Percentage of Ly9.1^{+} (CD44$^{-/-}$) cells in different thymic subsets in various chimeras. C. Ratio of Ly9.1^{+} cells in progressive T cell developmental stages. Data obtained from 3 groups of 4 chimeras each group reconstituted with 25%, 50% or 75% CD44$^{-/-}$ bone marrow. P values, 2-sample t test; *, P<0.05, **, P<0.01, ***, P<0.005 ****, P<0.001.

The DN stage can be broken down into four further stages of development. These four stages are usually referred to as DN1 to DN4, and are normally defined on the basis of $CD25^+$ and $CD44^+$ surface expression. However, as the cells we are investigating are $CD44^{-/-}$, an alternative marker, CD117, was utilised (254). CD117 has the same expression profile as CD44, that is: DN1= $CD44^+CD117^+CD25^-$, DN2= $CD44^+CD117^+CD25^+$, DN3= $CD44^-CD117^-CD25^+$, and DN4= $CD44^-CD117^-CD25^-$. Therefore as there appeared to be an increase in presence of $CD44^{-/-}$ cells in the double negative stage of T cell development, this stage was examined for the proportion of $CD44^{-/-}$ present in the various stages.

5.3.4.1. $CD44^{+/+}$ ($Ly5.2^+$) and $CD44^{-/-}$ ($Ly5.1^+$) donors → $CD44^{+/+}$ ($Ly5.2^+$) chimeras.

Chimeras were generated as described in section 3.3.1. The percentage of cells at each DN stage was examined for total, $Ly5.1^+$ and total $Ly5.1^-$ cells (Figure 5.9A). There was no a significant difference in the proportion of $Ly5.1^+$ cells among DN thymocytes. There was a greater contribution of $CD44^{-/-}$ cells to DN1 cells and lower contribution to DN2 cells (Figure 5.9B). Consequently, there was a lower DN2:DN3 ratio among $Ly5.1^+$ than $Ly5.2^+$ cells (Figure 5.9C).

5.3.4.2. $CD44^{+/+}$ ($Ly5.2^+$) and $CD44^{-/-}$ ($Ly5.1^+$) donors → $CD44^{-/-}$ ($Ly5.1^+$) chimeras.

To determine whether CD44 expression by the host influenced the T cell development during the DN stages, chimeras were generated in $CD44^{-/-}$ recipients as described in section 3.3.2.

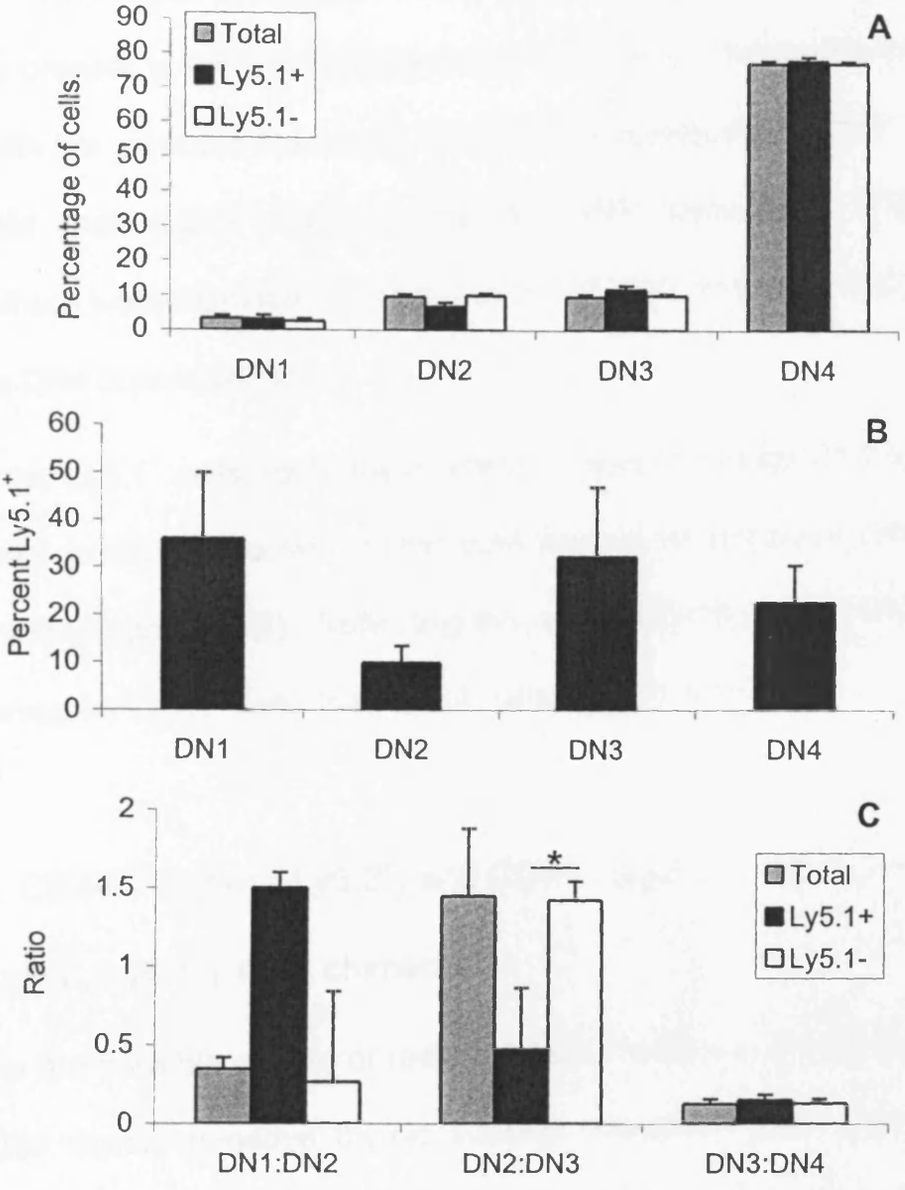

Figure 5.9. Analysis of double negative thymic subsets in $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly5.1^{+}$) → $CD44^{+/+}$ ($Ly5.2^{+}$) chimeras.

A. Distribution of total, $Ly5.1^{+}$ and $Ly5.1^{-}$ cells in different double negative thymic subsets. B. Percentage of $Ly5.1^{+}$ ($CD44^{-/-}$) cells in different double negative thymic subsets. C. Ratio of total, $Ly5.1^{+}$ and $Ly5.1^{-}$ cells in progressive T cell developmental stages. DN1= $CD117^{+}CD25^{-}$, DN2= $CD117^{+}CD25^{+}$, DN3= $CD117^{-}CD25^{+}$, DN4= $CD117^{-}CD25^{-}$. The data are mean ± s.e.m. from 2 chimeric groups of 3. P values, 2-sample t test; *, $P<0.05$.

The percentage of cells at each DN stage was examined for total, Ly5.1$^+$ and total Ly5.1$^-$ cells present in the DN subsets was determined. These differed from those obtained with the previous chimeras. There was a significantly higher proportion of Ly5.1$^+$ cells than Ly5.1$^-$ cells among the DN3 thymocytes (Figure 5.10A). Moreover there were significantly fewer Ly5.1$^+$ (CD44$^{-/-}$) cells than Ly5.2$^+$ (CD44$^{+/+}$) cells in the DN4 thymic subset.

In these mice Ly5.1$^+$ cells made the greatest contribution to the DN3 subset, while the relatively greater production of DN1 cells seen in the previous chimeras was not observed (Figure 5.10B). Reflecting this, a significantly higher DN3:DN4 ratio was observed for Ly5.1$^+$ cells than Ly5.1$^-$ cells (Figure 5.10C).

5.3.4.3. CD44$^{+/+}$ (Ly9.1$^-$, Ly5.2$^+$) and CD44$^{-/-}$ (Ly9.1$^+$, Ly5.1$^+$) → CD44$^{+/+}$ (Ly9.1$^-$, Ly5.1$^+$) 1100 chimeras

To reduce the possible effects of radioresistant T cells on the distribution of donor cells in the double negative thymic subsets, chimeras were generated using a higher dose of irradiation (1100 rads) as described in section 3.3.6. In addition, the Ly9 marker was used together with Ly5 to distinguish donor cells from any residual host T cells.

There was a significantly lower proportion of Ly9.1$^+$ (CD44$^{-/-}$) cells than Ly5.2$^+$ (CD44$^{+/+}$) cells in the DN2 populations, and relatively more Ly9.1$^+$ cells in DN3 populations (Figure 5.11A). There was also relatively more Ly9.1$^+$ cells in both the DN1 and DN3 subsets (Figure 5.11B). Significantly higher DN3:DN4 and lower DN2:DN3 ratios were observed for Ly9.1$^+$ cells (Figure 11C)

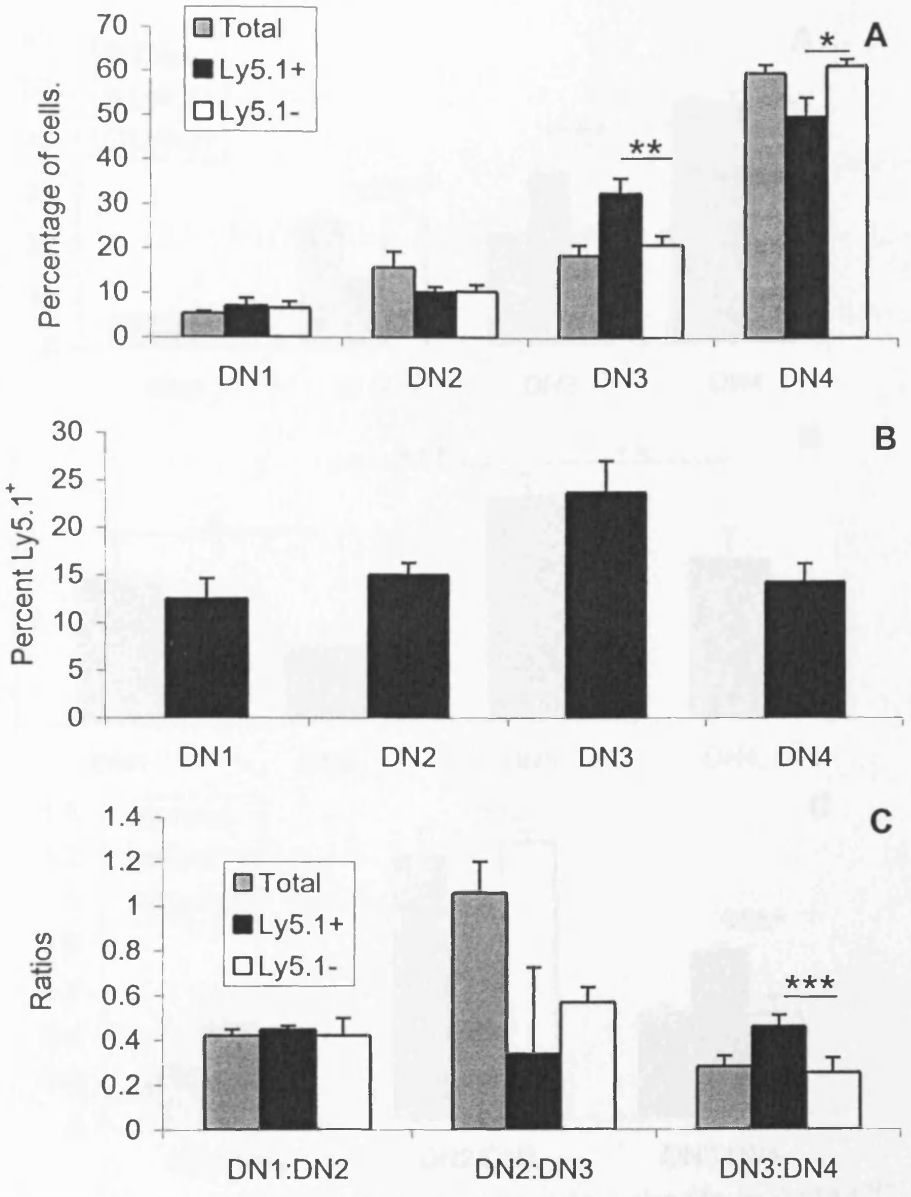

Figure 5.10. Analysis of double negative thymic subsets in $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly5.1^{+}$) → $CD44^{-/-}$ ($Ly5.1^{+}$) chimeras.

A. Distribution of total, $Ly5.1^{+}$ and $Ly5.1^{-}$ cells in different double negative thymic subsets. B. Percentage of $Ly5.1^{+}$ ($CD44^{-/-}$) cells in different DN thymic subsets. C. Ratio of total, $Ly5.1^{+}$ and $Ly5.1^{-}$ cells in progressive T cell developmental stages. The data are mean ± s.e.m. from 2 chimeric groups of 3. P values, 2-sample t test; *, $P<0.05$, ***, $P<0.005$.

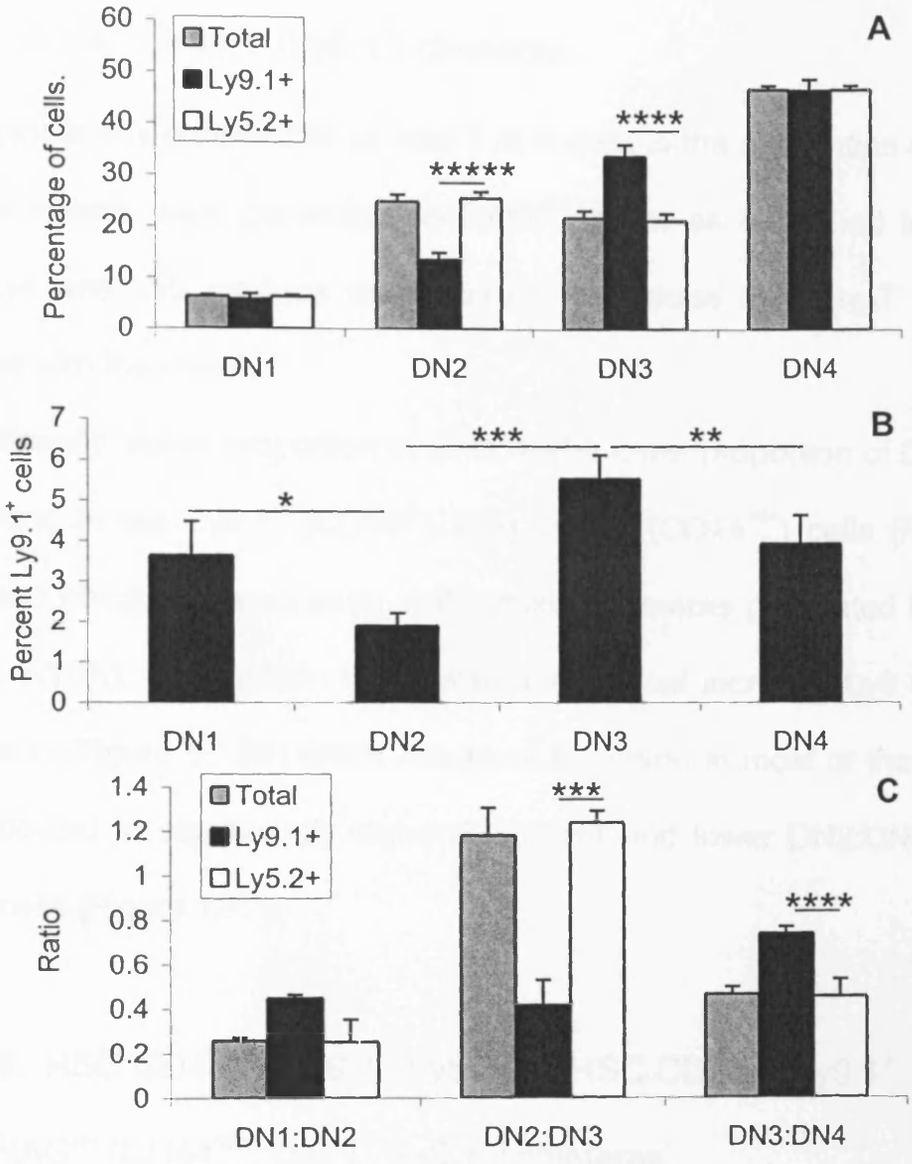

Figure 5.11. Analysis of double negative thymic subsets in CD44$^{+/+}$ (Ly5.2^{+}) & CD44$^{-/-}$ (Ly5.1^{+}) → CD44$^{-/-}$ (Ly5.1^{+}) 1100 chimeras.

A. Distribution of total, Ly9.1^{+} and Ly5.2^{+} cells in different double negative thymic subsets. B. Percentage of Ly9.1^{+} (CD44$^{-/-}$) cells in different double negative thymic subsets. C. Ratio of total, Ly9.1^{+} and Ly5.2^{+} cells in progressive T cell developmental stages. The data are mean ± s.e.m. from 2 chimeric groups of 4. P values, 2-sample t test; *, P<0.05, **, P<0.005, ****, P<0.001.

5.3.4.4. $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) and $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras

To eliminate any contribution of host T or B cells to the distribution of donor derived cells, chimeras were generated in $RAG2^{-/-}$ hosts as described in section 3.3.7. Both Ly9 and Ly5 markers were utilised to exclude host pre-T cells that could interfere with the results.

A significantly higher proportion of DN3, and a lower proportion of DN4 populations was found in the $Ly9.1^{+}$ ($CD44^{-/-}$) than $Ly5.2^{+}$ ($CD44^{+/+}$) cells (Figure 5.12A) a difference which was also seen in the mixed chimeras generated in $CD44^{-/-}$ hosts (Figure 5.10A). In addition there was a statistical increase $Ly9.1^{+}$ cells in DN3 population (Figure 5.12B) which has been observed in most of the chimeras. This was reflected in significantly higher DN3:DN4 and lower DN2:DN3 ratios for the $Ly9.1^{+}$ cells (Figure 12C).

5.3.4.5. HSC $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & HSC $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$) chimeras.

To eliminate the possibility that differing amounts of pre-T cells in the donor BM could alter the seeding balance of $CD44^{-/-}$ cells to $CD44^{+/+}$ cells in mixed chimeras, chimeras were generated using lineage depleted BM as described in section 3.3.9.

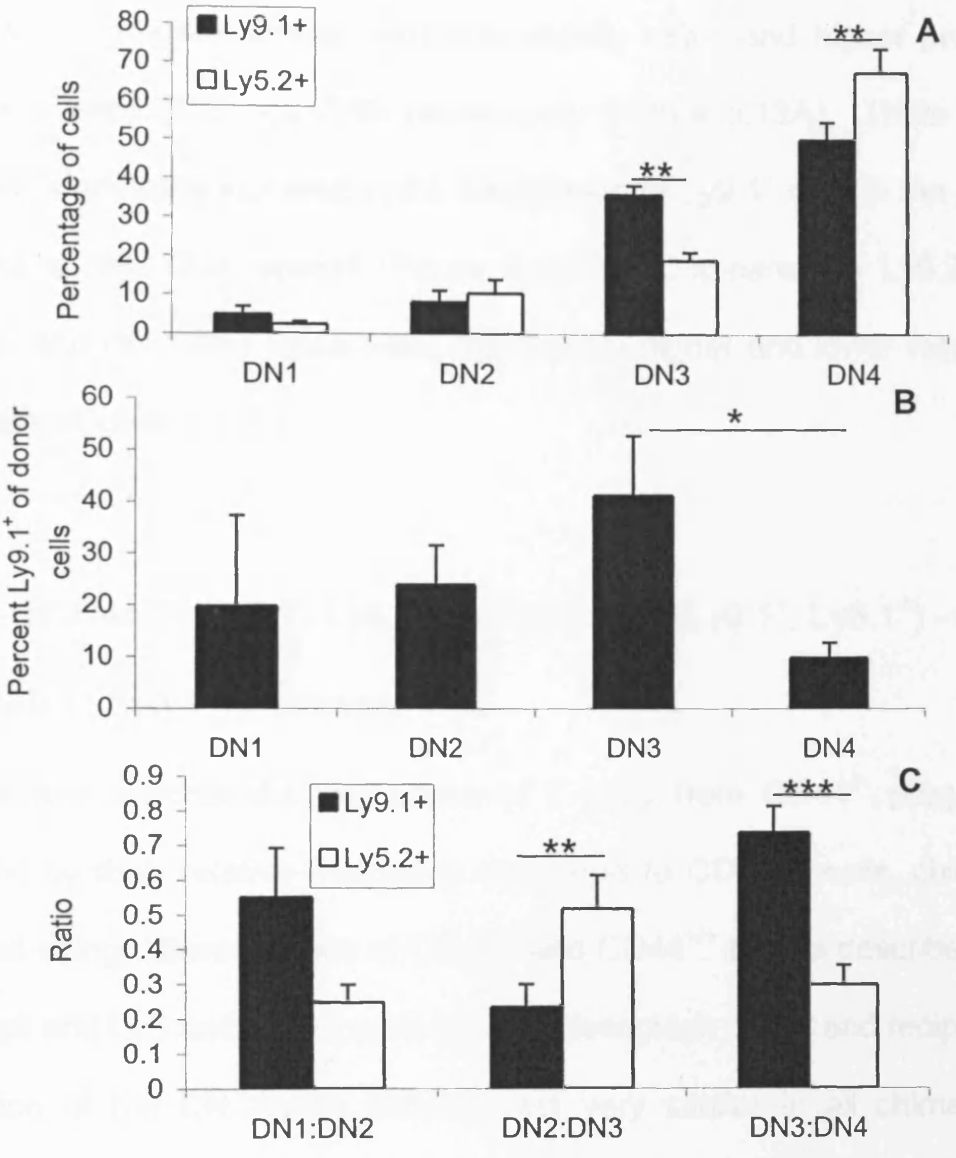

Figure 5.12. Analysis of double negative thymic subsets in CD44$^{+/+}$ (Ly9.1^{-}, Ly5.2^{+}) and CD44$^{-/-}$ (Ly9.1^{+}, Ly5.1^{+}) → RAG2$^{-/-}$ (CD44$^{+/+}$, Ly9.1^{-}, Ly5.1^{+}) chimeras

A. Distribution of Ly9.1^{+} and Ly5.2^{+} cells in different double negative thymic subsets. B. Percentage of Ly9.1^{+} (CD44$^{-/-}$) cells in different double negative thymic subsets. C. Ratio of Ly9.1^{+} and Ly5.2^{+} cells in progressive T cell developmental stages. The data are mean ± s.e.m. from 1 chimeric group of 4. P values, 2-sample t test; *, P<0.05, **, P<0.005, ***, P<0.005.

In these mice, the distribution of Ly9.1$^+$ and Ly5.2$^+$ cells among the DN thymic subsets was generally similar, although slightly lower and higher proportions of Ly9.1$^+$ cells were DN2 and DN3 respectively (Figure 5.13A). There was also a statistically significant increase in the percentage of Ly9.1$^+$ cells in the DN3 subset compared to the DN4 subset (Figure 5.13B). Compared to Ly5.2$^+$ cells, the DN1:DN2 and DN2:DN3 ratios were significantly higher and lower respectively for Ly9.1$^+$ cells (Figure 5.15C).

5.3.4.6. xCD44$^{+/+}$ (Ly9.1$^-$, Ly5.2$^+$) and yCD44$^{-/-}$ (Ly9.1$^+$, Ly5.1$^+$) → CD44$^{+/+}$ (Ly9.1$^-$, Ly5.1$^+$) chimeras.

To determine whether the production of T cells from CD44$^{-/-}$ progenitors was influenced by their relative frequency compared to CD44$^{+/+}$ cells, chimeras were generated using different ratios of CD44$^{-/-}$ and CD44$^{+/+}$ BM as described in section 3.3.8. Ly9 and Ly5 antibodies were used to distinguish donor and recipient cells. Distribution of the DN thymic subsets was very similar in all chimeras (Figure 5.14A), while the contribution of CD44$^{-/-}$ cells to each subset was proportional to the composition of the donor BM (Figure 5.14B). Consequently the ratios of cells found at different stages of progression were virtually identical (Figure 5.14C).

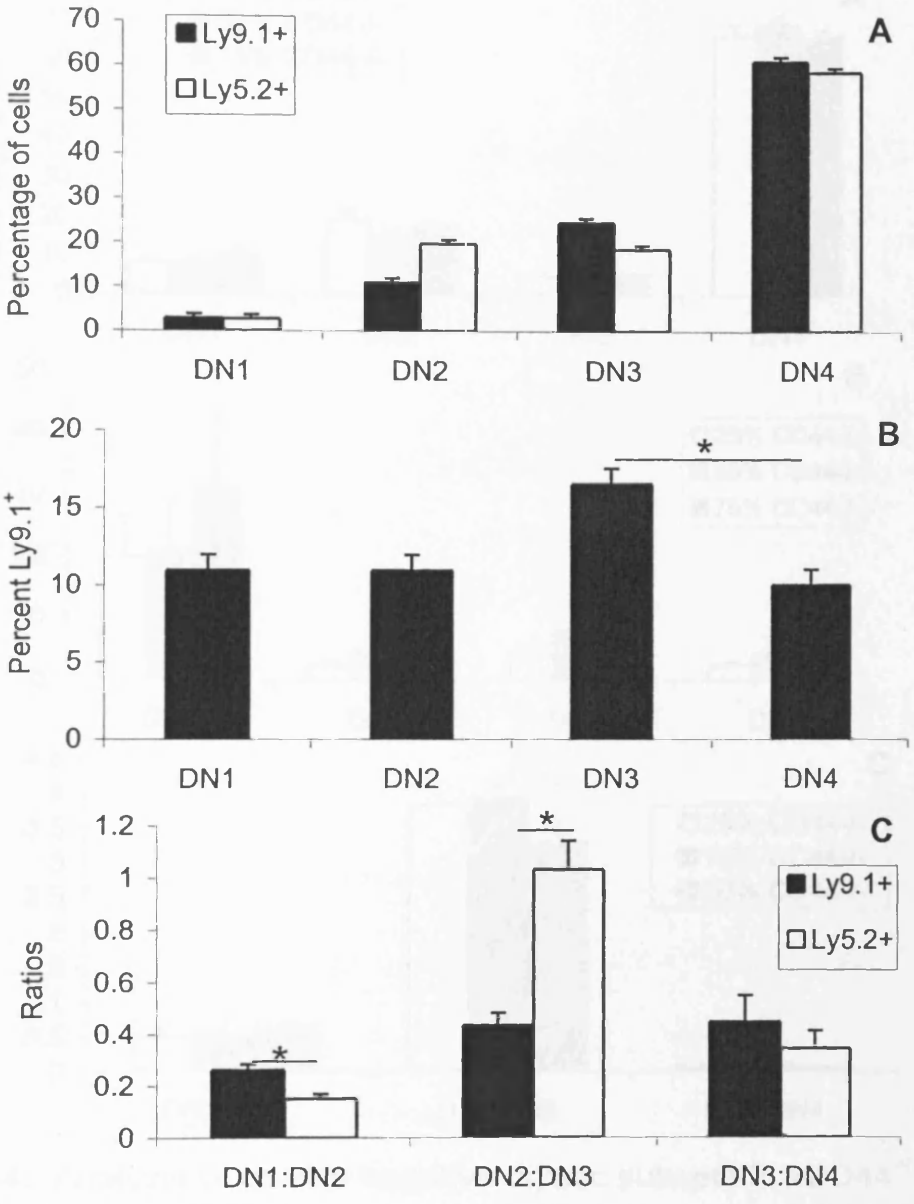

Figure 5.13. Analysis of double negative thymic subsets in HSC CD44$^{+/+}$ (Ly9.1^{-}, Ly5.2^{+}) & HSC CD44$^{-/-}$ (Ly9.1^{+}, Ly5.1^{+}) → RAG$^{-/-}$ (CD44$^{+/+}$, Ly9.1^{-}, Ly5.1^{+}) chimeras.

A. Distribution of Ly9.1^{+} and Ly5.2^{+} cells in different double negative thymic subsets. B. Percentage of Ly9.1^{+} (CD44$^{-/-}$) cells in different double negative thymic subsets. C. Ratio of Ly9.1^{+} and Ly5.2^{+} cells in progressive T cell developmental stages. The data are mean ± s.e.m. from 1 chimeric group of 4. P values, 2-sample t test; *, $P<0.05$.

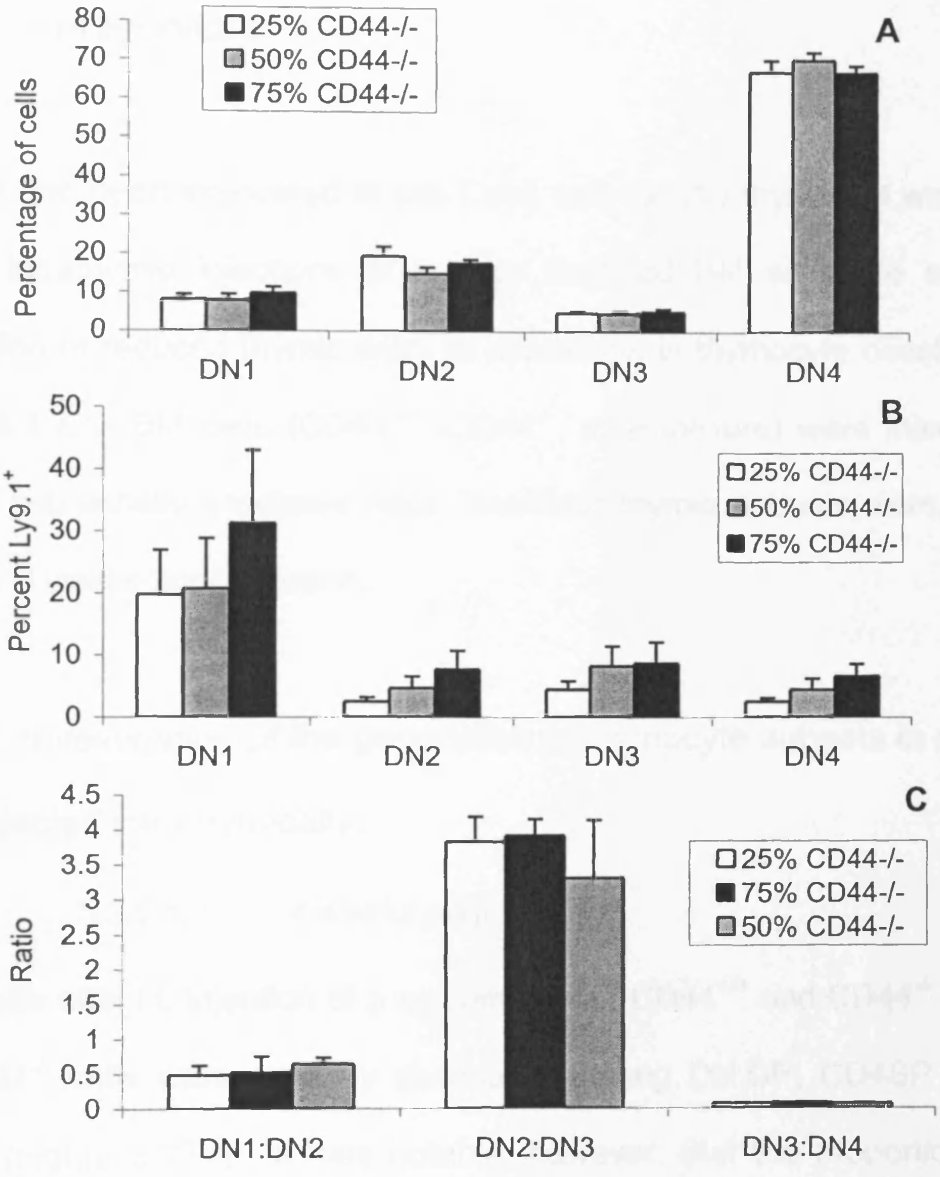

Figure 5.14. Analysis of double negative thymic subsets in xCD44$^{+/+}$ (Ly9.1^{-}, Ly5.2^{+}) and yCD44$^{-/-}$ (Ly9.1^{+}, Ly5.1^{+}) → CD44$^{+/+}$ (Ly9.1^{-}, Ly5.1^{+}) chimeras.

A. Distribution of Ly9.1^{+} and Ly5.2^{+} cells in different double negative thymic subsets. B. Percentage of Ly9.1^{+} (CD44$^{-/-}$) cells in different double negative thymic subsets. C. Ratio of of Ly9.1^{+} and Ly5.2^{+} cells in progressive T cell developmental stages. Data obtained from 3 groups of 4 chimeras each group reconstituted with either 25%, 50% or 75% CD44$^{-/-}$ bone marrow.

5.3.5. T cell development following intrathymic injection of progenitors

As CD44 has been implicated in pre-T cell entry to the thymus, it was decided to perform intrathymic injections of lineage depleted BM so as to eliminate any contribution of reduced thymic entry to alterations in thymocyte development (see section 4.3.2.). BM cells ($CD44^{+/+}$, $CD44^{-/-}$, or a mixture) were injected into the thymi of sub-lethally irradiated mice, and their thymic subsets were examined 4 weeks or 6 weeks post-injection.

5.3.5.1. Investigation of the generation of thymocyte subsets in mice injected intrathymically.

5.3.5.1.1. 4 weeks post injection.

Four weeks after i.t. injection of a combination of $CD44^{+/+}$ and $CD44^{-/-}$ BM, $CD44^{+/+}$ and $CD44^{-/-}$ cells were similarly distributed among DN,DP, CD4SP and CD8SP subsets (Figure 5.15A). It was notable, however, that the proportion of $CD44^{-/-}$ thymocytes was less than 30%, despite injecting equal numbers of $CD44^{+/+}$ and $CD44^{-/-}$ progenitors (Figure 15B). Thus the deficit in producing thymocytes from $CD44^{-/-}$ progenitors was not strictly linked to reduced thymic homing.

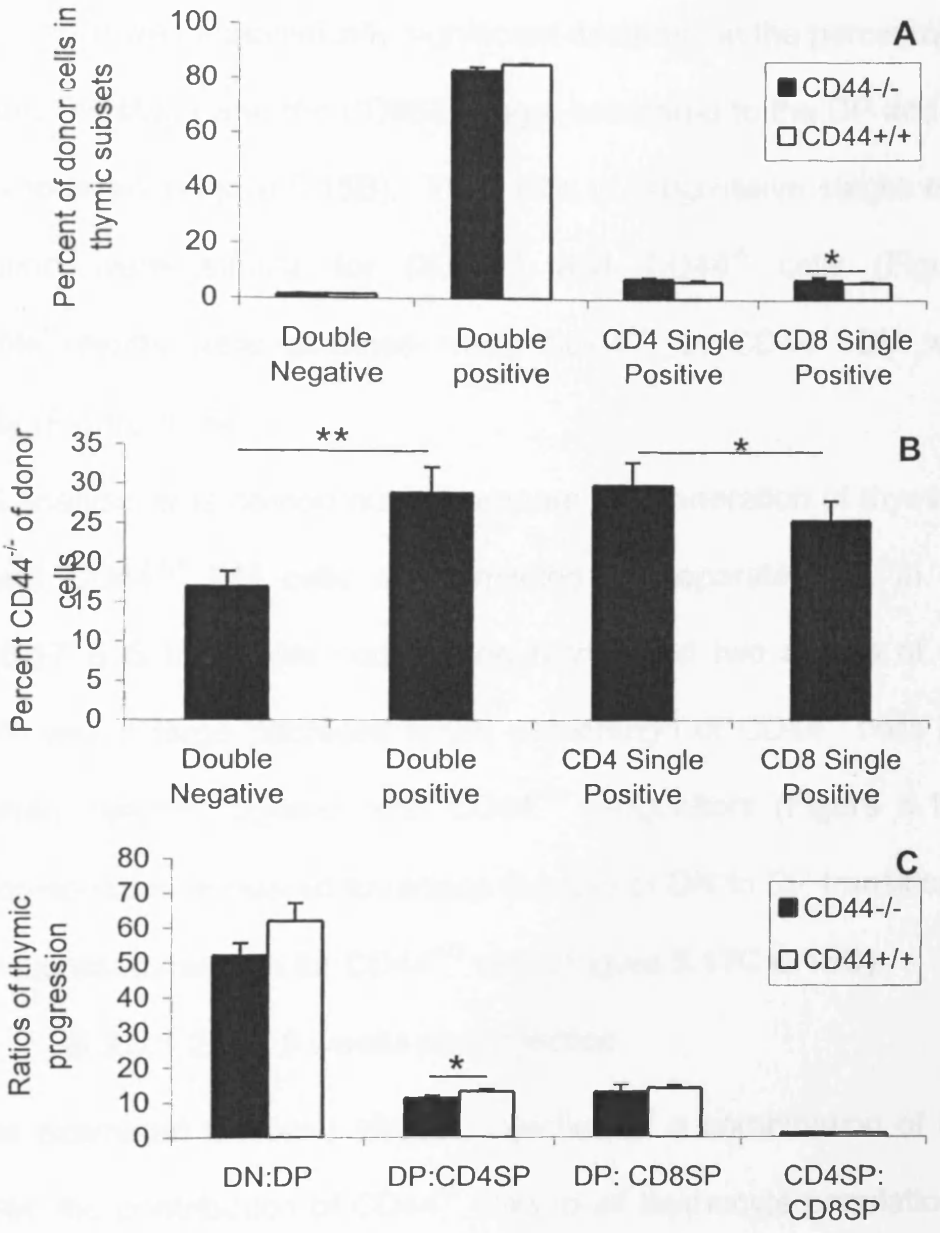

Figure 5.15. Analysis of thymic subsets in chimeras generated after intrathymic injection of $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$) progenitors 4 weeks post injection.

A. Distribution of in $CD44^{+/+}$ and $CD44^{-/-}$ cells in different thymic subsets. B. Percentage contribution of $CD44^{-/-}$ of donor cells in different thymic subsets. C. Ratio of distribution of $CD44^{+/+}$ and $CD44^{-/-}$ cells in progressive T cell developmental stages DN, Double Negative. DP, Double Positive. SP, Single Positive. The data are mean ± s.e.m. from 1 chimeric group of 5. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$.

In addition, there was a statistically significant decrease in the percentage of $CD44^{-/-}$ cells in the DN stage and the CD8SP stage, compared to the DP and the CD4SP stages respectively (Figure 5.15B). The ratios of progressive stages of thymocyte differentiation were similar for $CD44^{+/+}$ and $CD44^{-/-}$ cells (Figure 5.15C). Comparable results were obtained when $CD44^{+/+}$ or $CD44^{-/-}$ BM was injected individually (Figure 5.16).

Statistical analysis was carried out to compare the generation of thymocytes when $CD44^{-/-}$ and $CD44^{+/+}$ BM cells were injected i.t. separately vs. in combination (Figures 5.17 & 5.18). This comparison highlighted two effects of competition. First, there was a large decrease in the percentage of $CD44^{-/-}$ cells in all thymic stages when injected together with $CD44^{+/+}$ progenitors (Figure 5.17B & 18B). Second, competition appeared to reduce the rate of DN to DP transition for $CD44^{-/-}$ cells, and increase this rate for $CD44^{+/+}$ cells (Figure 5.17C & 18C).

5.3.5.1.2. 6 weeks post injection.

Mice were examined 6 weeks after i.t. injection of a combination of $CD44^{+/+}$ and $CD44^{-/-}$ BM, the contribution of $CD44^{-/-}$ cells to all thymocyte populations remained low (<25%) (Figure 19B). At this time, there was a slight decrease in the percentage of $CD44^{-/-}$ cells in the CD8SP stage (Figure 5.19A). This was reflected in a higher CD4SP:CD8SP ratio for $CD44^{-/-}$ cells (Figure 5.19C).

When mice injected i.t. with $CD44^{+/+}$ or $CD44^{-/-}$ BM individually were compared six weeks post-injection, there were significantly higher percentages of $CD44^{-/-}$ cells in the DP and the CD4SP thymic stages compared to the donor $CD44^{+/+}$ cells, but a decrease in the percentage of $CD44^{-/-}$ CD8SP cells (Figure 5.20A).

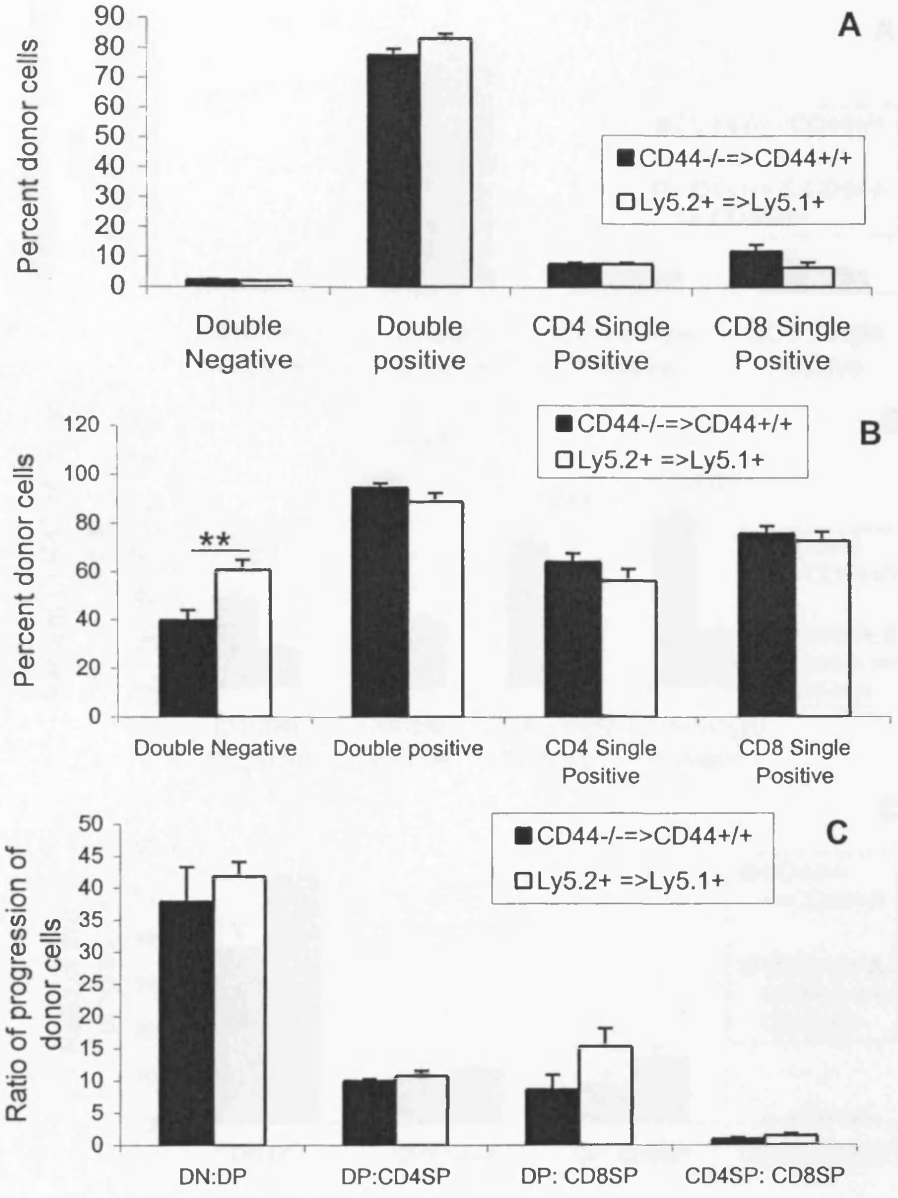

Figure 5.16. Analysis of thymic subsets in chimeras generated after intrathymic injection of $CD44^{+/+}$ ($Ly5.2^{+}$) or $CD44^{-/-}$ ($Ly9.1^{+}$) progenitors 4 weeks post injection.

A. Distribution of $Ly9.1^{+}$ or $Ly5.2^{+}$ cells in different thymic subsets. B. Percentage contribution of $Ly9.1^{+}$ and $Ly5.2^{+}$ cells in different thymic subsets. C. Ratio of progression of $Ly9.1^{+}$ and $Ly5.2^{+}$ cells in progressive T cell developmental stages. DN, Double Negative. DP, Double Positive. SP, Single Positive. The data are mean ± s.e.m. from 2 chimeric groups of 4. P values, 2-sample t test; **, $P<0.01$.

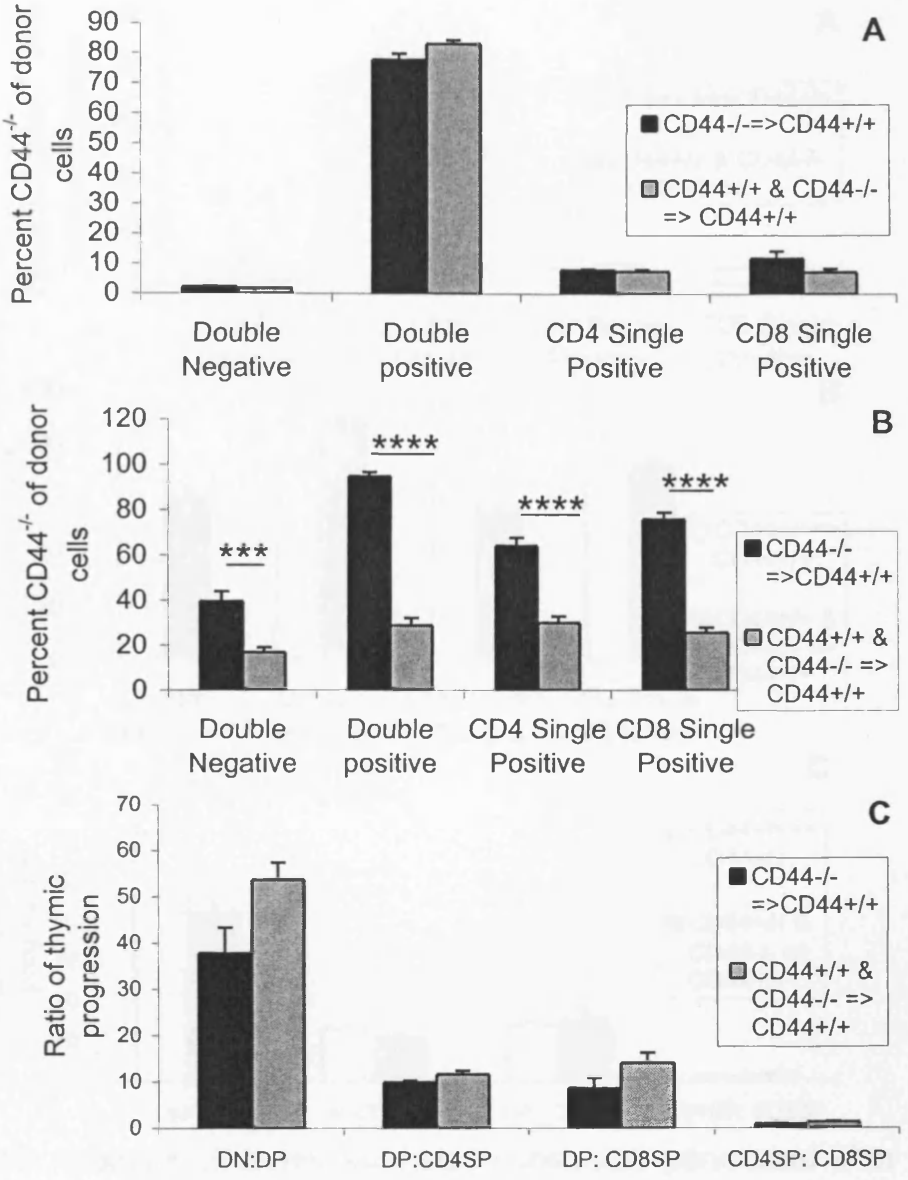

Figure 5.17. Analysis of thymic subsets in chimeras generated after intrathymic injection of $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$) or $CD44^{-/-}$ only progenitors 4 weeks post injection.

A. Distribution of $CD44^{-/-}$ cells in different thymic subsets. B. Percentage contribution of $CD44^{-/-}$ cells in different thymic subsets. C. Ratio of progression of $CD44^{-/-}$ cells in progressive T cell developmental stages. DN, Double Negative. DP, Double Positive. SP, Single Positive. The data are mean ± s.e.m. from 1 chimeric group of 4, reconstituted with $CD44^{-/-}$ bone marrow and 1 chimeric group of 5, reconstituted with $CD44^{-/-}$ and $CD44^{+/+}$ bone marrow. P values, 2-sample t test; ***, $P<0.005$, ****, $P<0.001$.

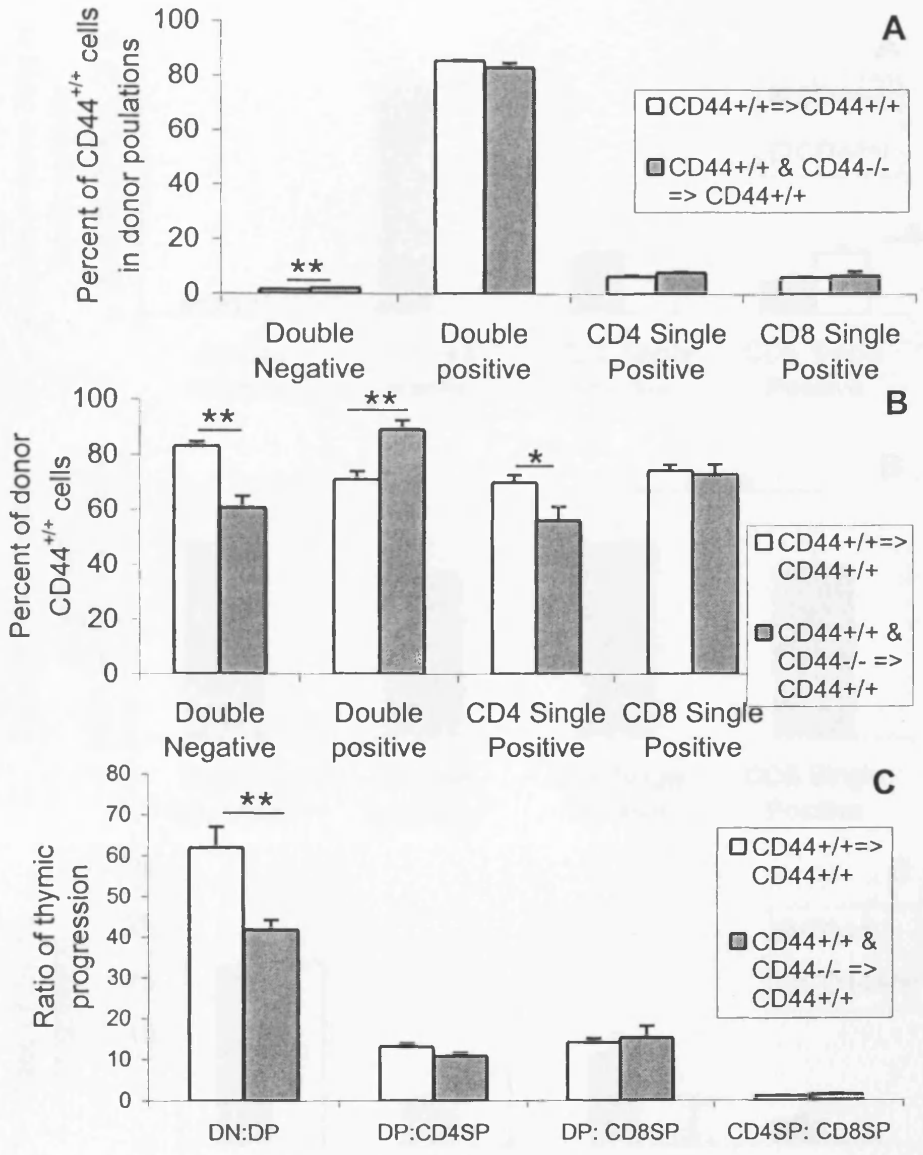

Figure 5.18. Analysis of thymic subsets in chimeras generated after intrathymic injection of $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$) or $CD44^{+/+}$ only progenitors 4 weeks post injection.

A. Distribution of donor $CD44^{+/+}$ cells in different donor thymic subsets. B. Percentage contribution of donor $CD44^{+/+}$ cells in different donor thymic subsets. C. Ratio of progression of donor $CD44^{+/+}$ cells in progressive T cell developmental stages. DN, Double Negative. DP, Double Positive. SP, Single Positive. The data are mean ± s.e.m. from 1 chimeric group of 4, reconstituted with $CD44^{-/-}$ bone marrow and 1 chimeric group of 5, reconstituted with $CD44^{-/-}$ and $CD44^{+/+}$ bone marrow. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$.

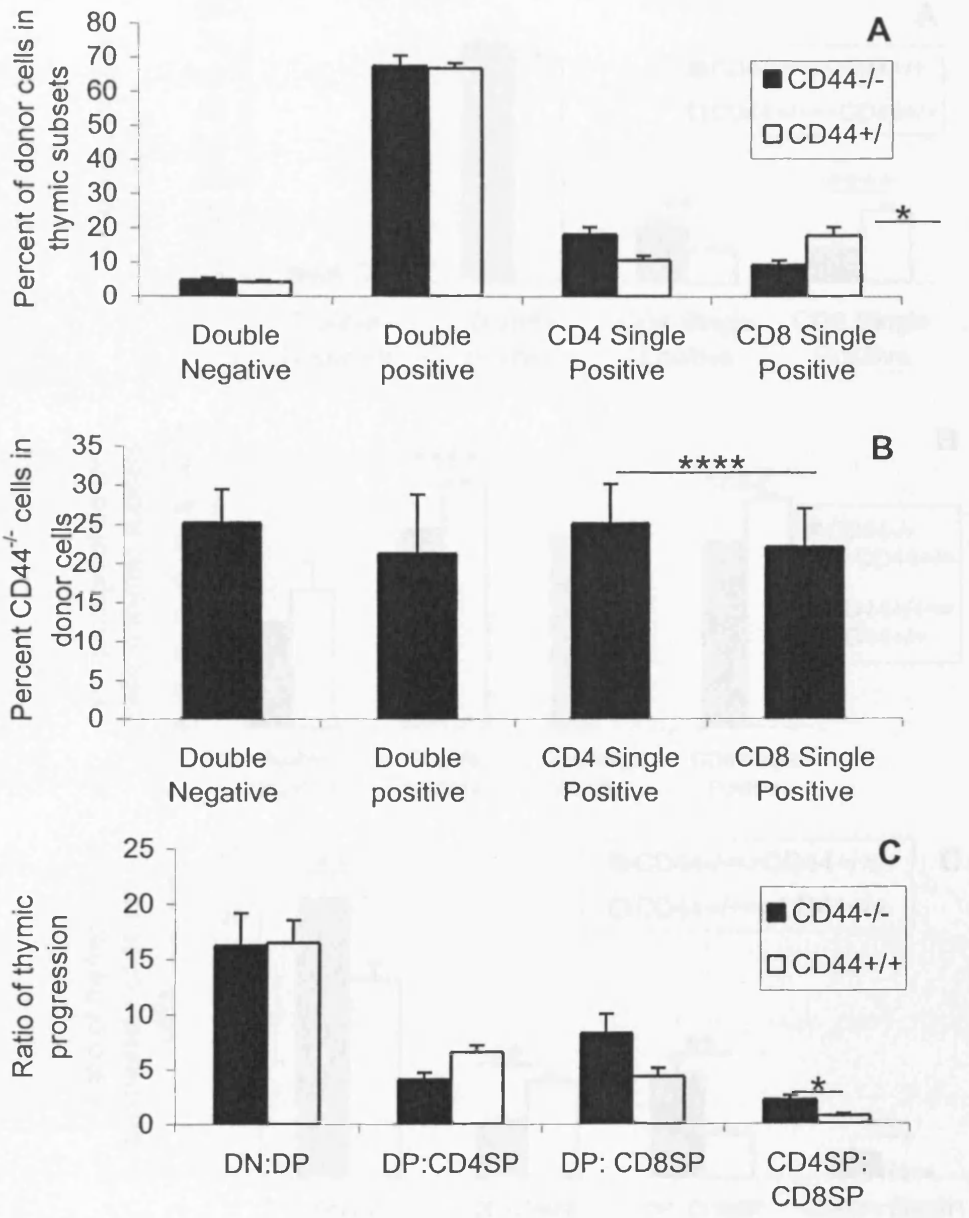

Figure 5.19. Analysis of thymic subsets in chimeras generated after intrathymic injection of $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$) progenitors 6 weeks post injection.

A. Distribution of in $CD44^{+/+}$ and $CD44^{-/-}$ cells in different thymic subsets. B. Percentage contribution of $CD44^{-/-}$ of donor cells in different thymic subsets. C. Ratio of distribution of $CD44^{+/+}$ and $CD44^{-/-}$ cells in progressive T cell developmental stages DN, Double Negative. DP, Double Positive. SP, Single Positive. The data are mean ± s.e.m. from 1 chimeric group of 5. P values, 2-sample t test; *, $P<0.05$, ****, $P<0.001$.

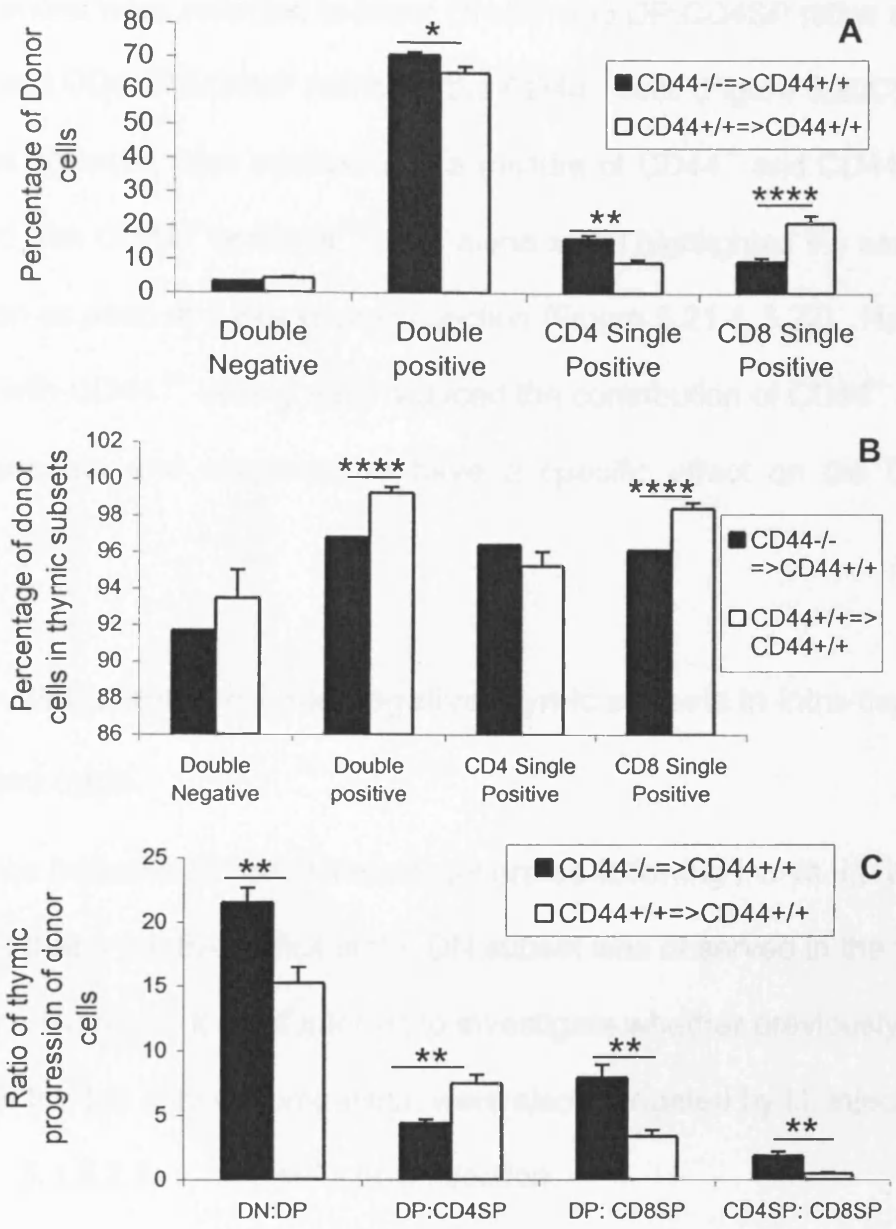

Figure 5.20. Analysis of thymic subsets in chimeras generated after intrathymic injection of $CD44^{+/+}$ ($Ly5.2^{+}$) or $CD44^{-/-}$ ($Ly9.1^{+}$) progenitors 6 weeks post injection.

A. Distribution of $CD44^{+/+}$ and $CD44^{-/-}$ cells in different thymic subsets. B. Percentage contribution of $CD44^{+/+}$ and $CD44^{-/-}$ cells in different thymic subsets. C. Ratio of progression $CD44^{+/+}$ and $CD44^{-/-}$ cells in progressive T cell developmental stages. DN, Double Negative. DP, Double Positive. SP, Single Positive. The data are mean ± s.e.m. from 2 chimeric groups of 4. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ****, $P<0.001$.

These differences were reflected in lower DN:DP and DP:CD4SP ratios and higher DP:CD8SP and CD4SP:CD8SP ratios for the $CD44^{-/-}$ cells (Figure 5.20C). Comparisons between mice injected with a mixture of $CD44^{-/-}$ and $CD44^{+/+}$ BM vs. mice injected with $CD44^{-/-}$ or $CD44^{+/+}$ cells alone again highlighted the same effects of competition as seen at 4 weeks post injection (Figure 5.21 & 5.22). Namely, that competition with $CD44^{+/+}$ cells greatly reduced the contribution of $CD44^{-/-}$ cells to all thymocyte subsets and appeared to have a specific effect on the DN to DP transition.

5.3.5.2. Investigation of double negative thymic subsets in intra-thymically injected mice.

One difference between mixed chimeras generated following i.v. vs. i.t. injection of BM cells was that a relative deficit in the DN subset was observed in the former but not the latter. Therefore it as of interest to investigate whether previously observed differences in the DN subset competition were also abrogated by i.t. injection.

5.3.5.2.1. 4 weeks post injection.

Distribution of $CD44^{-/-}$ cells among DN thymocytes in i.t. injected mice is shown in Figures 5.23 and 5.24. The results are differ substantially from those in i.v. injected mice (e.g. Figure 5.9). In particular, the relative contribution of $CD44^{-/-}$ cells to DN1 vs. DN2 subsets was reversed, so that in the i.t. chimeras the DN:DN2 and DN2:DN3 ratios were lower and higher respectively for $CD44^{-/-}$ than $CD44^{+/+}$ cells. This was true whether BM cells were injected as a mixture or individually (Figures 5.23 -5.26)

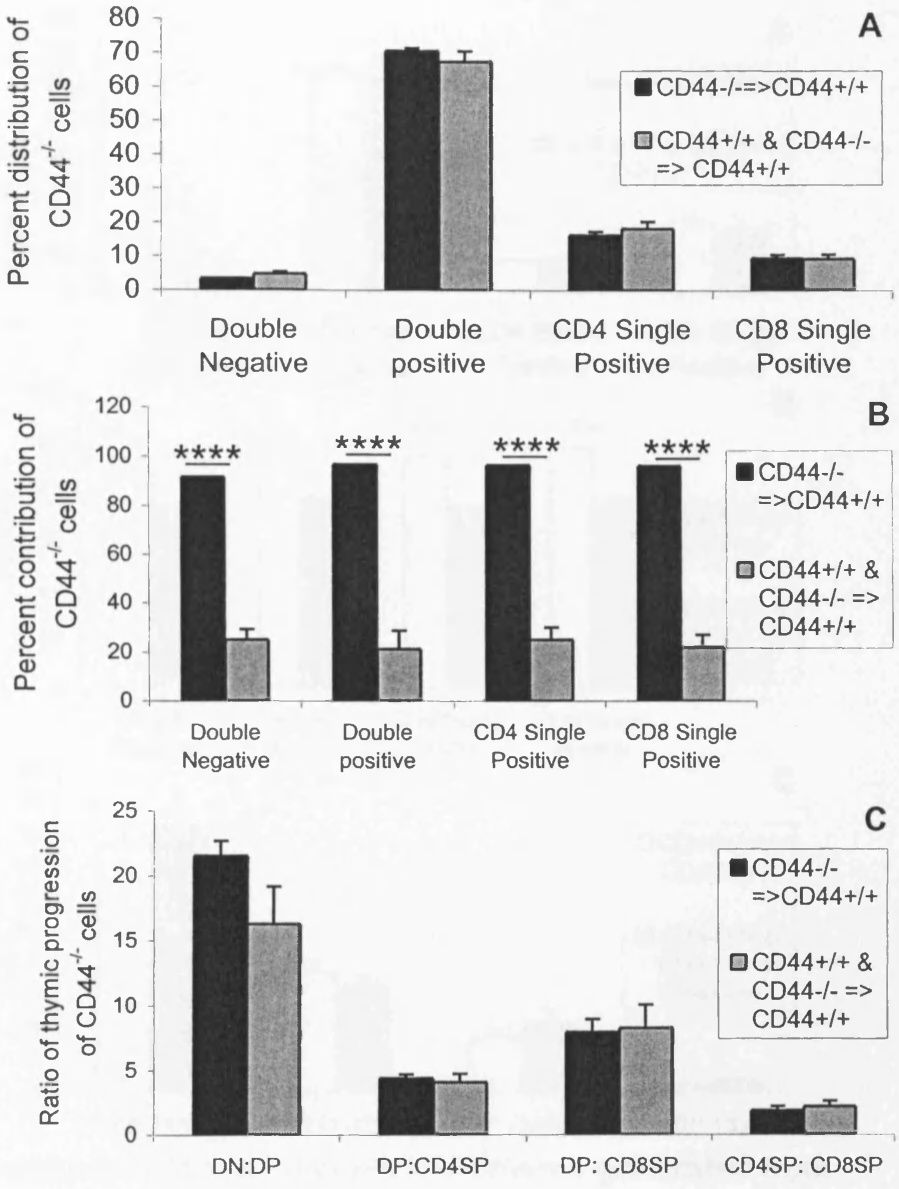

Figure 5.21. Analysis of thymic subsets in chimeras generated from intrathymic injection of $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$) or $CD44^{-/-}$ only progenitors 6 weeks post injection.

A. Distribution of $CD44^{-/-}$ cells in different thymic subsets. B. Percentage contribution of $CD44^{-/-}$ cells in different thymic subsets. C. Ratio of progression of $CD44^{-/-}$ cells in progressive T cell developmental stages. DN, Double Negative. DP, Double Positive. SP, Single Positive. The data are mean ± s.e.m. from 1 chimeric group of 4, reconstituted with $CD44^{-/-}$ bone marrow and 1 chimeric group of 5, reconstituted with $CD44^{-/-}$ and $CD44^{+/+}$ bone marrow. P values, 2-sample t test; ***, $P<0.005$, ****, $P<0.001$.

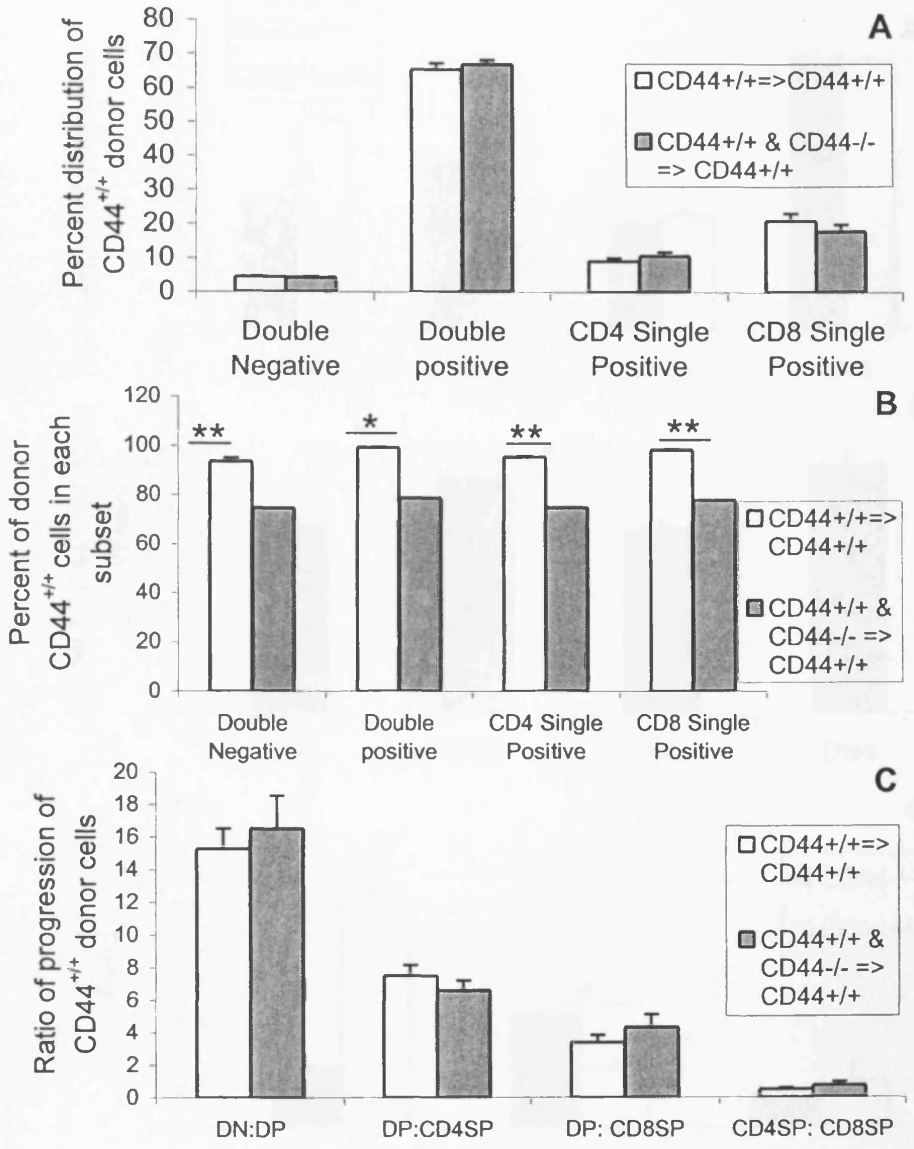

Figure 5.22. Analysis of thymic subsets in chimeras generated from intrathymic injection of $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$) or $CD44^{+/+}$ only progenitors 6 weeks post injection.

A. Distribution of donor $CD44^{+/+}$ cells in different thymic subsets. B. Percentage contribution of donor $CD44^{+/+}$ cells in different thymic subsets. C. Ratio of progression of donor $CD44^{+/+}$ cells in progressive T cell developmental stages. DN, Double Negative. DP, Double Positive. SP, Single Positive. The data are mean ± s.e.m. from 1 chimeric group of 4, reconstituted with $CD44^{-/-}$ bone marrow and 1 chimeric group of 5, reconstituted with $CD44^{-/-}$ and $CD44^{+/+}$ bone marrow. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$.

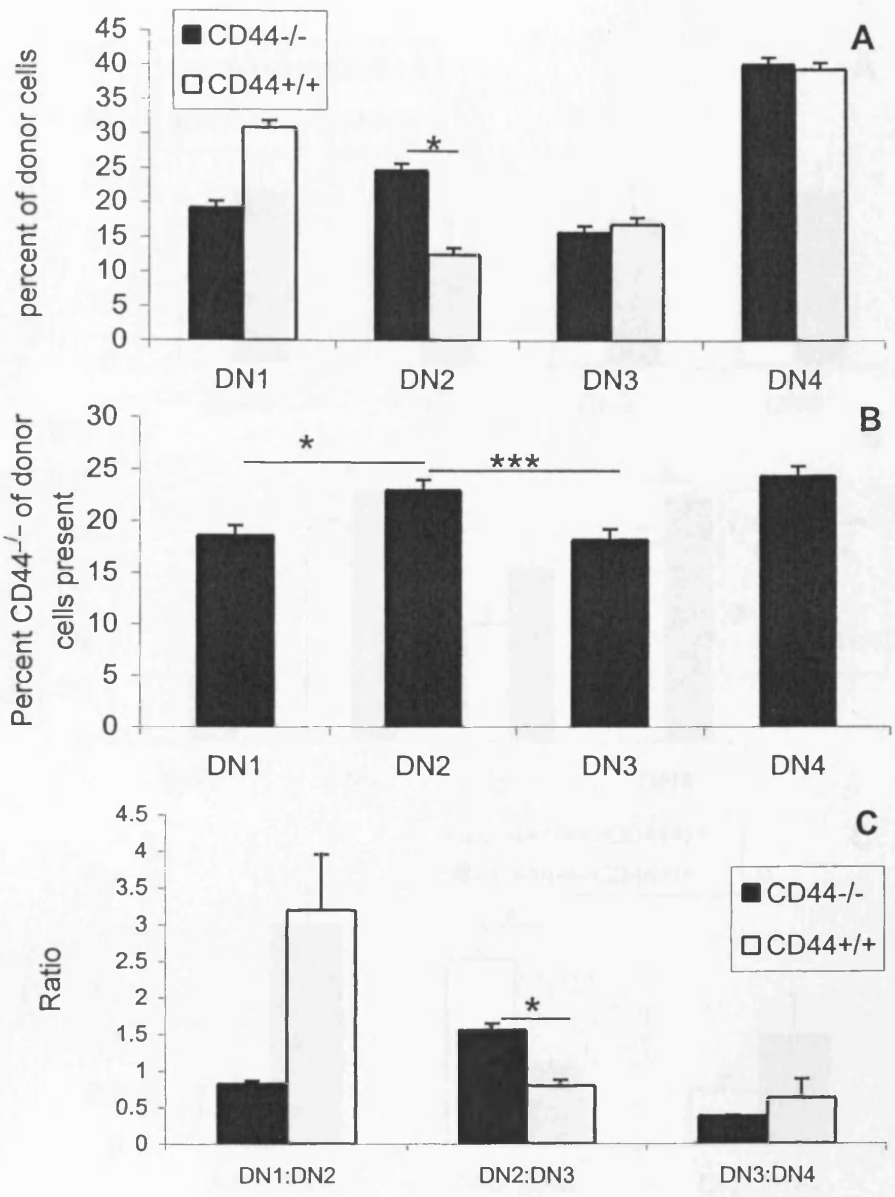

Figure 5.23. Analysis of DN thymic subsets in chimeras generated from intrathymic injection of $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$) progenitors 4 weeks post injection.

A. Distribution of in $CD44^{+/+}$ and $CD44^{-/-}$ cells in different double negative thymic subsets. B. Percentage of $CD44^{-/-}$ of donor cells in different double negative thymic subsets. C. Ratio of of $CD44^{+/+}$ and $CD44^{-/-}$ cells in progressive T cell developmental stages. DN1= $CD117^{+}CD25^{-}$, DN2= $CD117^{+}$ $CD25^{+}$, DN3= $CD117^{-}CD25^{+}$, DN4= $CD117^{-}CD25^{-}$. The data are mean ± s.e.m. from 1 chimeric group of 5. P values, 2-sample t test; *, $P<0.05$, ***, $P<0.005$.

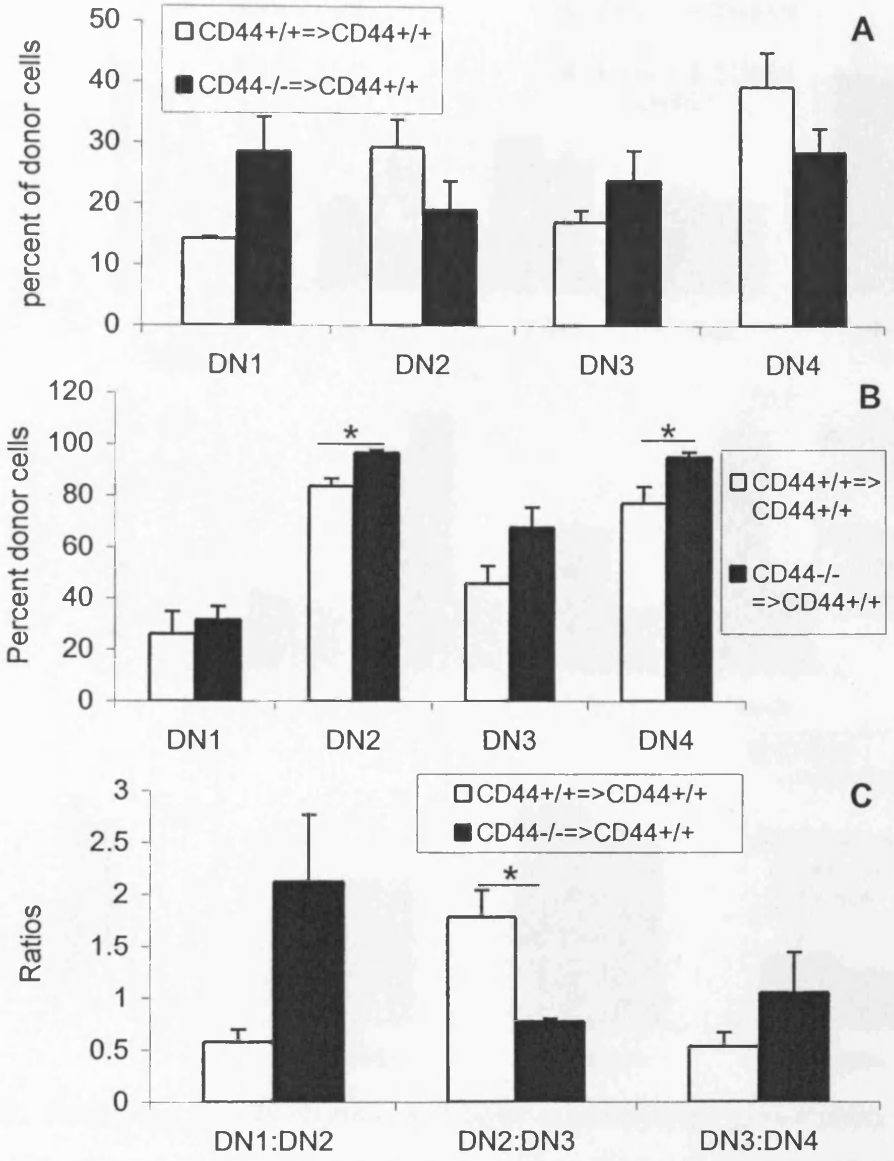

Figure 5.24. Analysis of DN thymic subsets in chimeras generated from intrathymic injections of $CD44^{+/+}$ ($Ly5.2^{+}$) or $CD44^{-/-}$ ($Ly9.1^{+}$) progenitors chimeras 4 weeks post injection.

A. Distribution of $CD44^{+/+}$ and $CD44^{-/-}$ cells in different double negative thymic subsets. B. Percentage $CD44^{+/+}$ and $CD44^{-/-}$ cells in different thymic subsets. C. Ratio of $CD44^{+/+}$ and $CD44^{-/-}$ cells in progressive T cell developmental stages. The data are mean ± s.e.m. from 2 chimeric groups of 4. P values, 2-sample t test; *, $P<0.05$.

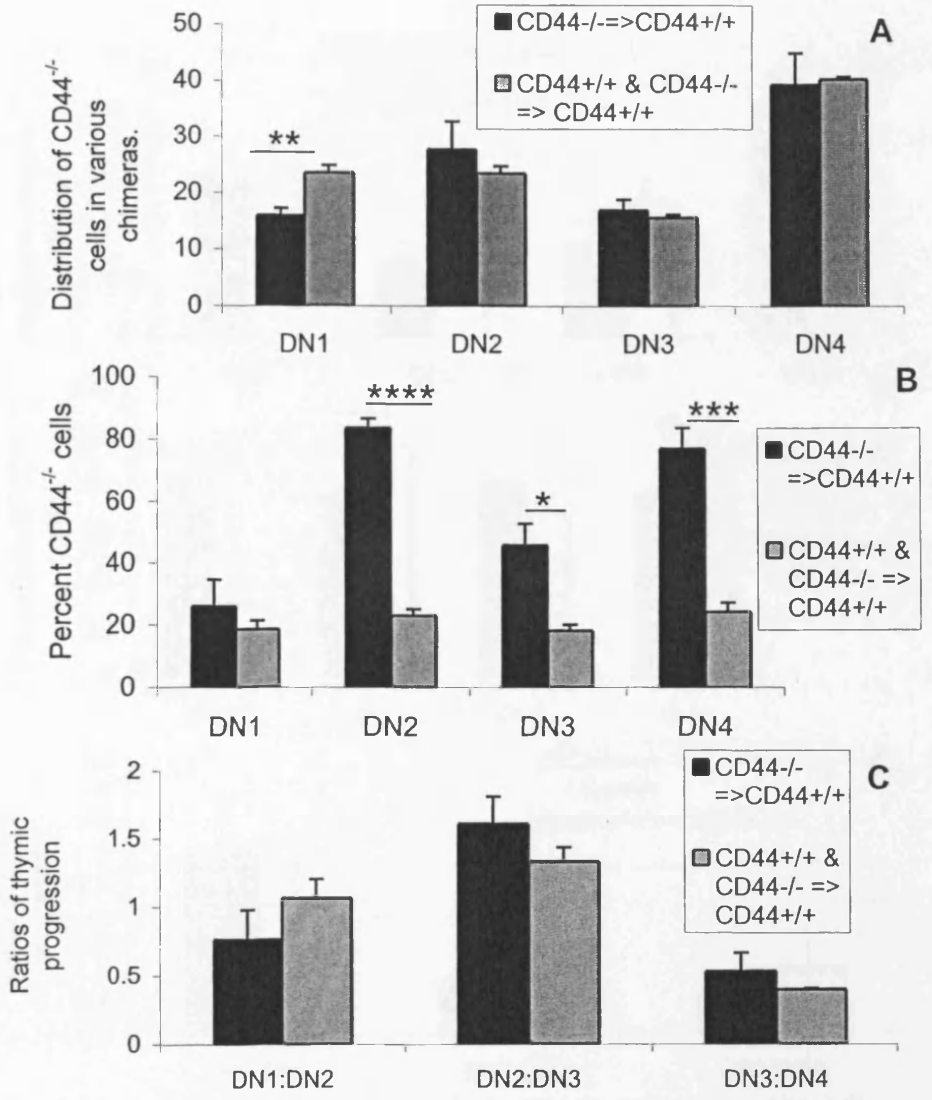

Figure 5.25. Analysis of DN thymic subsets in chimeras generated from intrathymic injections of $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$) or $CD44^{-/-}$ only progenitors 4 weeks post injection.

A. Distribution of $CD44^{-/-}$ cells in different double negative thymic subsets. B. Percentage contribution of $CD44^{-/-}$ cells in different double negative thymic subsets. C. Ratio of progression of $CD44^{-/-}$ cells in progressive T cell developmental stages. The data are mean ± s.e.m. from 1 chimeric group of 4, reconstituted with $CD44^{-/-}$ bone marrow and 1 chimeric group of 5, reconstituted with $CD44^{-/-}$ and $CD44^{+/+}$ bone marrow. P values, 2-sample t test; ***, $P<0.005$, ****, $P<0.001$.

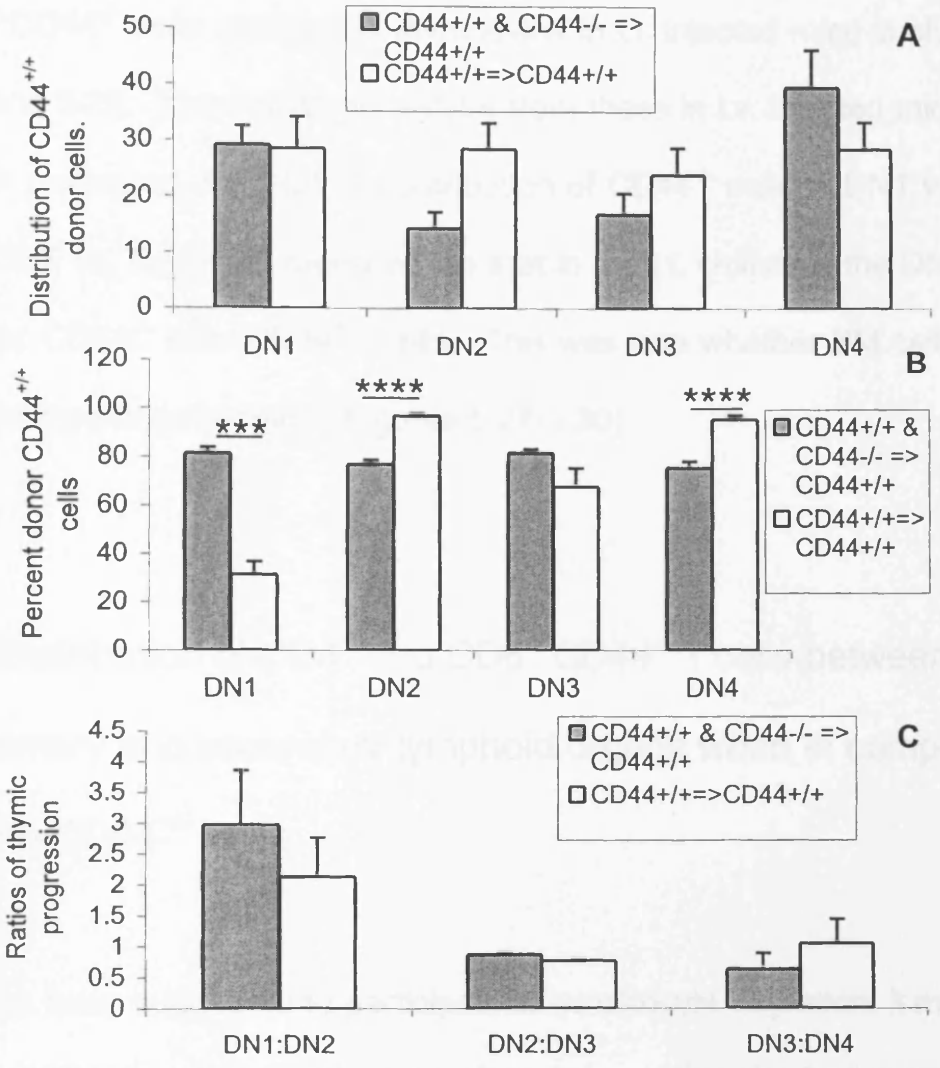

Figure 5.26. Analysis of DN thymic subsets in chimeras generated from $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$) or $CD44^{+/+}$ only progenitors 4 weeks post injection.

A. Distribution of donor $CD44^{+/+}$ cells in different donor double negative thymic subsets. B. Percentage contribution of donor $CD44^{+/+}$ cells in different donor double negative thymic subsets. C. Ratio of progression of donor $CD44^{+/+}$ cells in progressive T cell developmental stages. The data are mean ± s.e.m. from 1 chimeric group of 4, reconstituted with $CD44^{-/-}$ bone marrow and 1 chimeric group of 5, reconstituted with $CD44^{-/-}$ and $CD44^{+/+}$ bone marrow. P values, 2-sample t test; ***, $P<0.005$, ****, $P<0.001$.

5.3.5.2.2. 6 weeks post injection.

Distribution of $CD44^{-/-}$ cells among DN thymocytes in i.t. injected mice is shown in Figures 5.27 and 5.28. The results are similar from those in i.v. injected mice (e.g. Figure 5.9). In particular, the relative contribution of $CD44^{-/-}$ cells to DN1 vs. DN2 subsets, and DN3 vs. DN4 was repeated, so that in the i.t. chimeras the DN2:DN3 ratios higher for $CD44^{-/-}$ than $CD44^{+/+}$ cells. This was true whether BM cells were injected as a mixture or individually (Figures 5.27-5.30)

5.3.6. Distribution of $CD4^+$ and $CD8^+$ $CD44^{-/-}$ T cells between primary and secondary lymphoid organs when in competition with $CD44^{+/+}$ cells.

Since CD44 has been suggested to participate in lymphocyte migration, it might be expected that $CD44^{-/-}$ and $CD44^{+/+}$ lymphocytes would distribute differently between tissues when in direct competition. To determine if this is the case, the ratio of $CD44^{+/+}$ to $CD44^{-/-}$ T cells and B cells was compared in different lymphoid and non-lymphoid tissues.

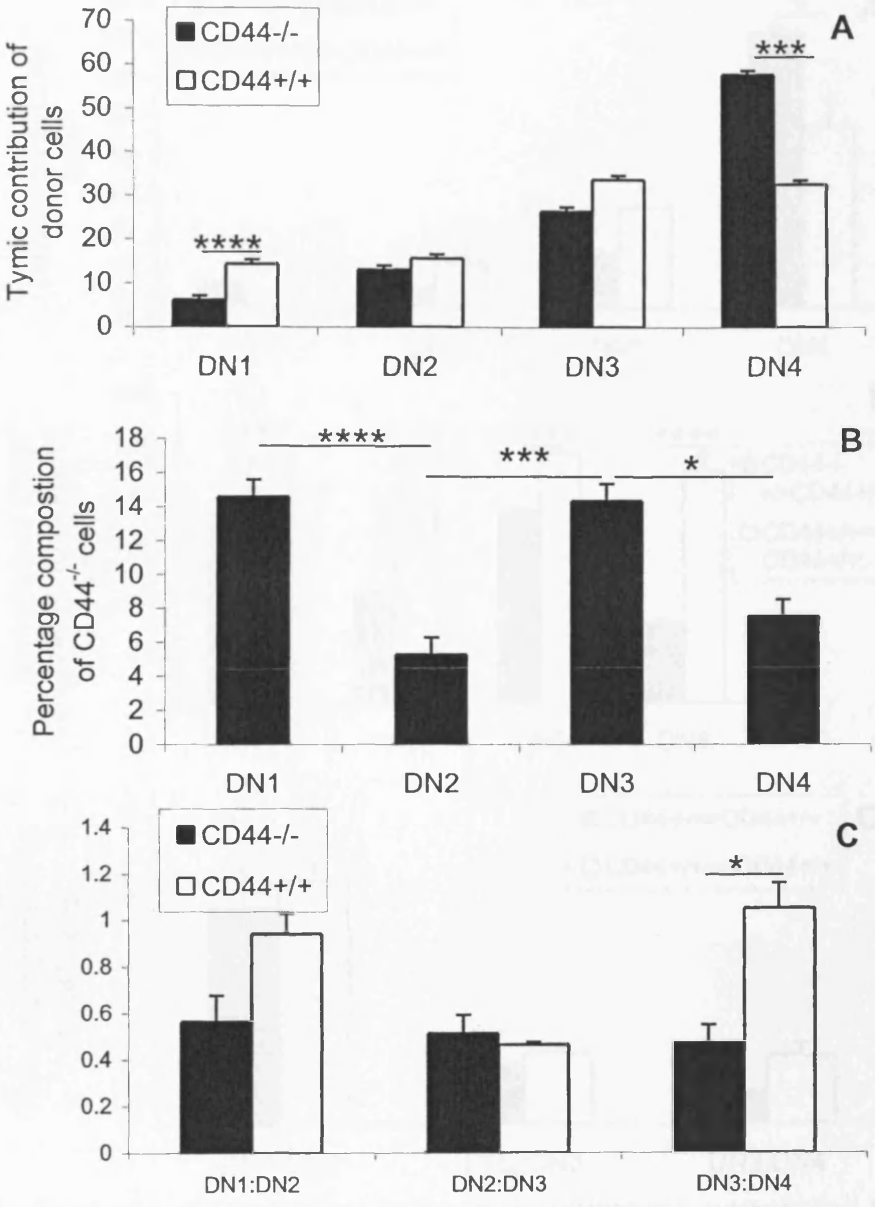

Figure 5.27. Analysis of DN thymic subsets in chimeras generated form intrathymic injections $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$) progenitors 6 weeks post injection.

A. Distribution of in $CD44^{+/+}$ and $CD44^{-/-}$ cells in different double negative thymic subsets. B. Percentage contribution of $CD44^{-/-}$ of donor cells in different double negative donor thymic subsets. C. Ratio of distribution of $CD44^{+/+}$ and $CD44^{-/-}$ cells in progressive T cell developmental. The data are mean ± s.e.m. from 1 chimeric group of 5. P values, 2-sample t test; *, $P<0.05$, ***, $P<0.005$, ****, $P<0.001$.

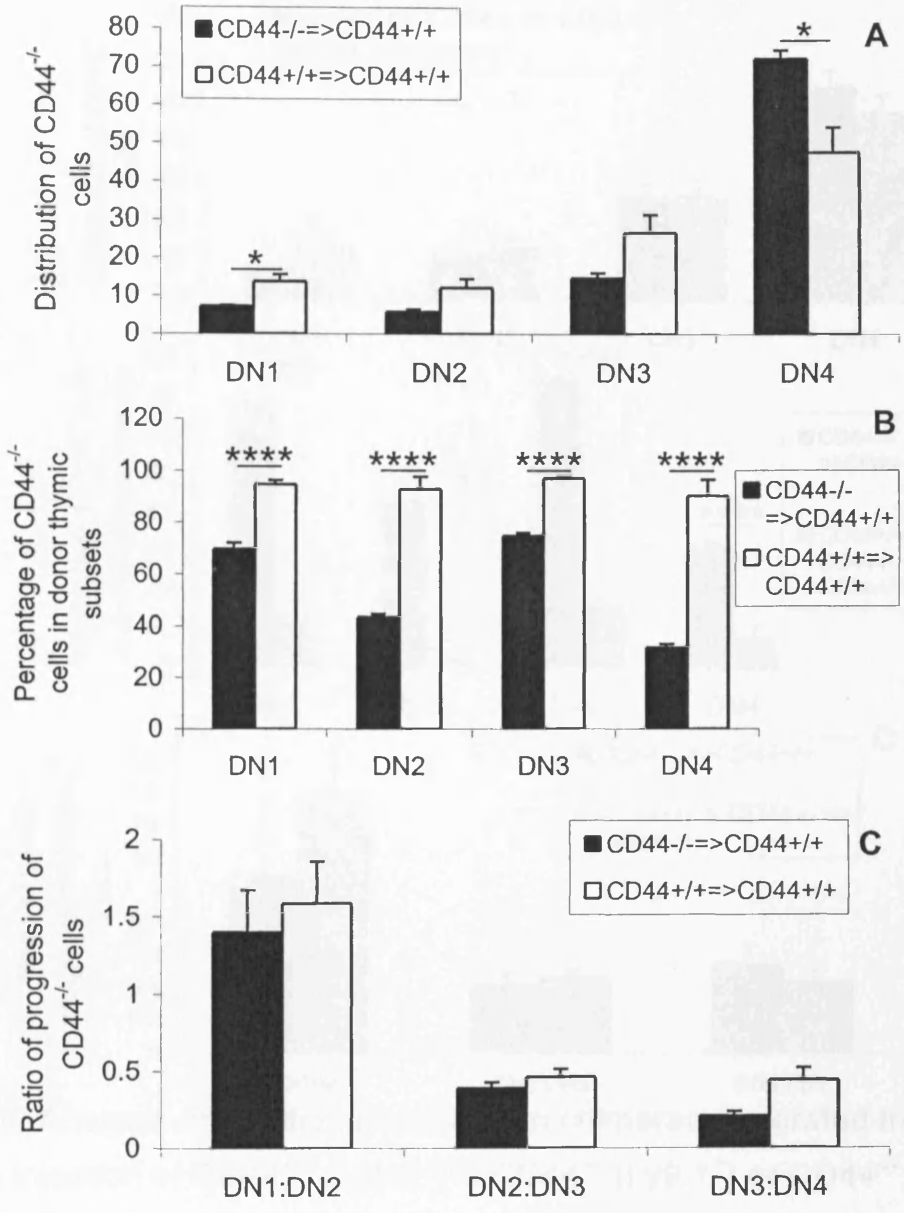

Figure 5.28. Analysis of DN thymic subsets in chimeras generated from intrathymic injections of $CD44^{+/+}$ ($Ly5.2^{+}$) or $CD44^{-/-}$ ($Ly9.1^{+}$) progenitors 6 weeks post injection.

A. Distribution of $CD44^{+/+}$ and $CD44^{-/-}$ cells in different double negative thymic subsets. B. Percentage contribution of $CD44^{+/+}$ and $CD44^{-/-}$ cells in different double negative thymic subsets. C. Ratio of progression $CD44^{+/+}$ and $CD44^{-/-}$ cells in progressive T cell developmental stages. The data are mean ± s.e.m. from 2 chimeric groups of 4. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ****, $P<0.001$.

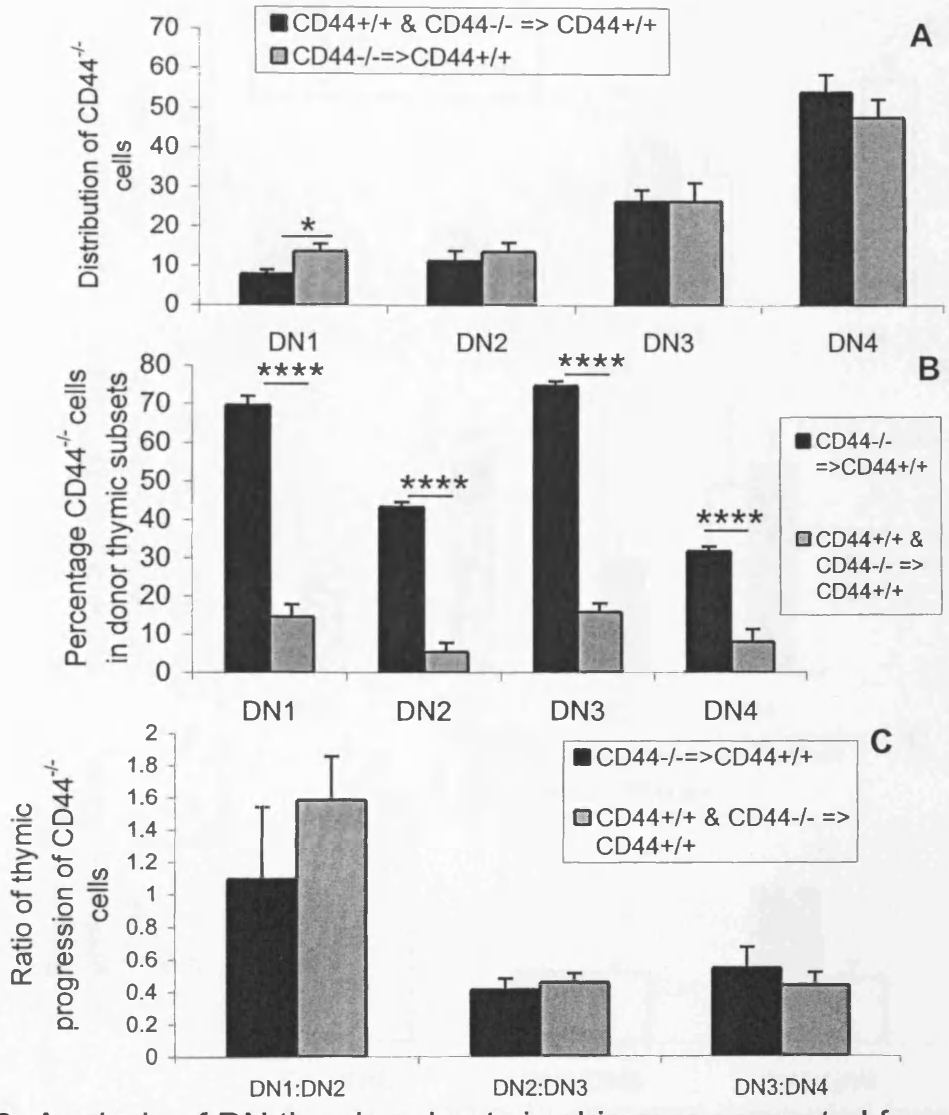

Figure 5.29. Analysis of DN thymic subsets in chimeras generated from intrathymic injection of $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$) or $CD44^{-/-}$ only progenitors 6 weeks post injection.

A. Distribution of $CD44^{-/-}$ cells in different double negative thymic subsets. B. Percentage contribution of $CD44^{-/-}$ cells in different double negative thymic subsets. C. Ratio of progression of $CD44^{-/-}$ cells in progressive T cell developmental stages. The data are mean ± s.e.m. from 1 chimeric group of 4, reconstituted with $CD44^{-/-}$ bone marrow and 1 chimeric group of 5, reconstituted with $CD44^{-/-}$ and $CD44^{+/+}$ bone marrow. P values, 2-sample t test; *, $P<0.05$, ****, $P<0.001$

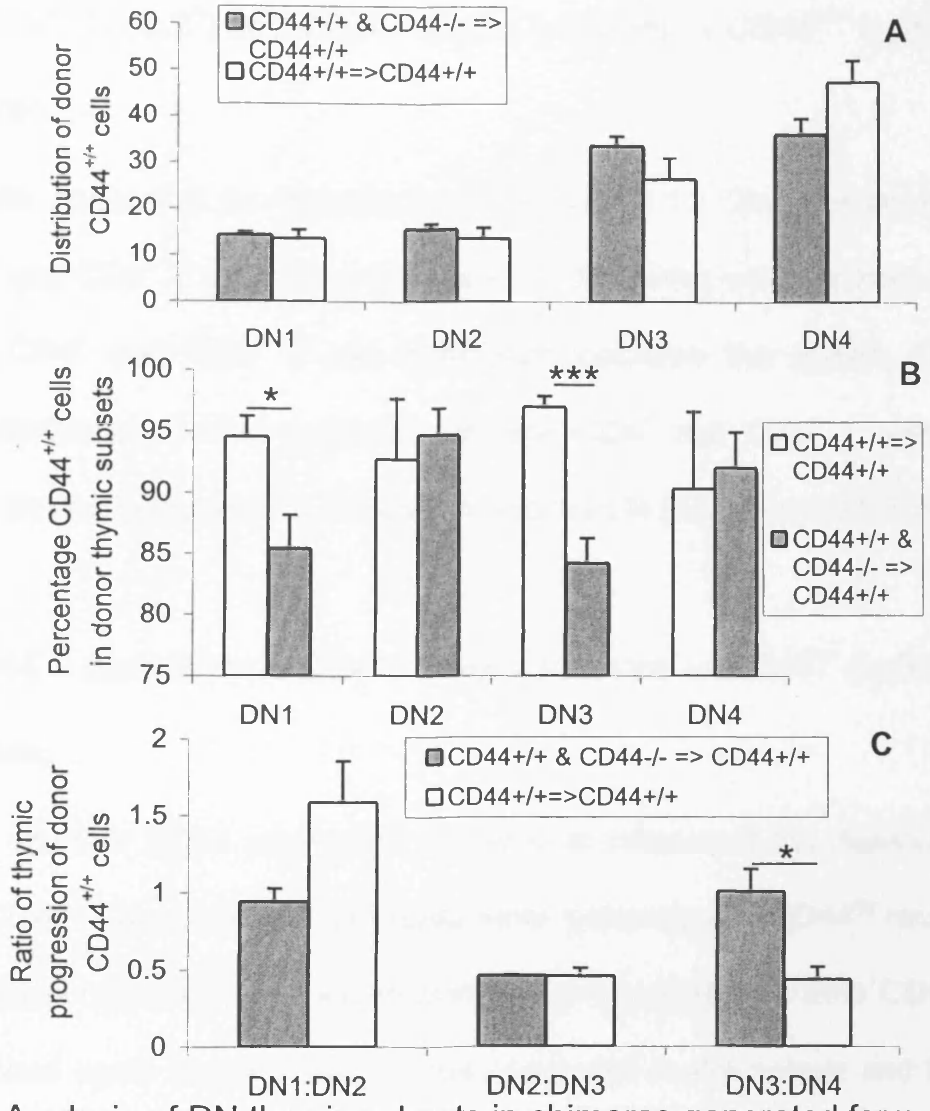

Figure 5.30. Analysis of DN thymic subsets in chimeras generated form intrathymic injections of $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly9.1^{+}$) or $CD44^{+/+}$ only progenitors 6 weeks post injection.

A. Distribution of donor $CD44^{+/+}$ cells in different double negative thymic subsets. B. Percentage contribution of donor $CD44^{+/+}$ cells in different double negative thymic subsets. C. Ratio of progression of donor $CD44^{+/+}$ cells in progressive T cell developmental stages. The data are mean ± s.e.m. from 1 chimeric group of 4, reconstituted with $CD44^{-/-}$ bone marrow and 1 chimeric group of 5, reconstituted with $CD44^{-/-}$ and $CD44^{+/+}$ bone marrow. P values, 2-sample t test; *, $P<0.05$, ***, $P<0.005$.

5.3.6.1. $CD44^{+/+}$ ($Ly5.2^{+}$) and $CD44^{-/-}$ ($Ly5.1^{+}$) donors → $CD44^{+/+}$ ($Ly5.2^{+}$) chimeras.

Chimeras were generated as described in section 3.3.1. The percentages of $CD44^{-/-}$ $CD4^{+}$ and $CD8^{+}$ T cells were compared to determine whether there was a difference in $CD4^{+}$ and $CD8^{+}$ T cell distribution between the spleen, LN and thymus. A significantly lower proportion of both $CD4^{+}$ and $CD8^{+}$ T cells were $CD44^{-/-}$ in the thymus compared to the spleen and the LN (Figures 5.31A & B).

5.3.6.2. $CD44^{+/+}$ ($Ly5.2^{+}$) and $CD44^{-/-}$ ($Ly5.1^{+}$) donors → $CD44^{-/-}$ ($Ly5.1^{+}$) chimeras.

To determine whether CD44 expression by the host influenced the distribution of $CD44^{-/-}$ and $CD44^{+/+}$ lymphocytes, chimeras were generated in $CD44^{-/-}$ recipients as described in section 3.3.2. A significantly lower proportion of both $CD4^{+}$ and $CD8^{+}$ T cells was again found in the thymus compared to the spleen and the LN (Figures 5.31C & D).

5.3.6.3. $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) and $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) → $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.1^{+}$) 1100 chimeras

To reduce the possible effects of radioresistant T cells on the distribution of donor cells, chimeras were also generated using a higher dose of irradiation (1100 rads) as described in section 3.3.6. In addition, the Ly9 marker was used together with Ly5 to distinguish donor cells from any residual host T cells.

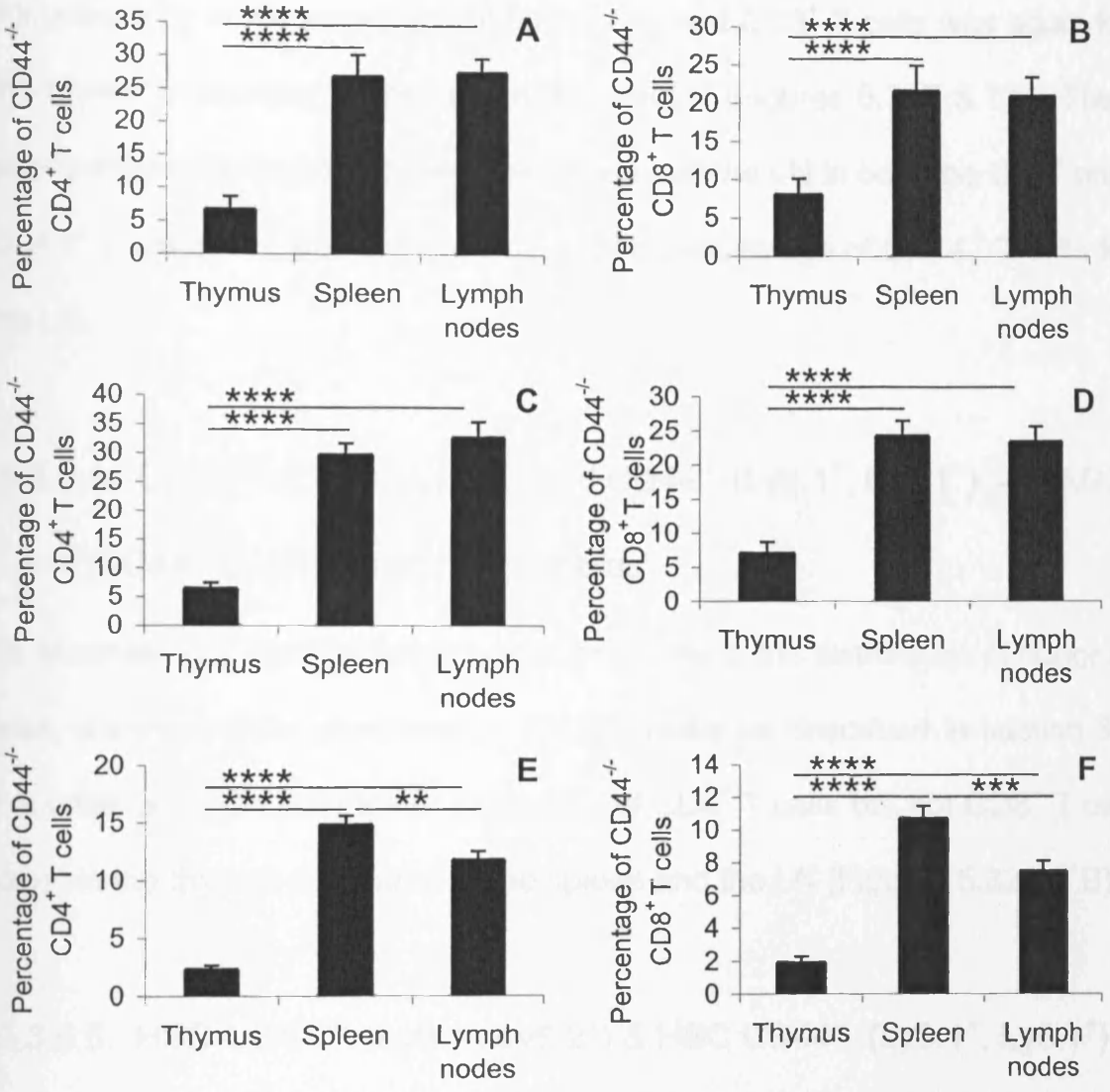

Figure 5.31. Analysis of the percentage of $CD4^+$ and $CD8^+$ $CD44^{-/-}$ cells in the thymus, spleen and lymph nodes of various chimeras, I.

A, B. $CD44^{+/+}$ ($Ly5.2^+$) & $CD44^{-/-}$ ($Ly5.1^+$) → $CD44^{+/+}$ ($Ly5.2^+$). C, D. $CD44^{+/+}$ ($Ly5.2^+$) & $CD44^{-/-}$ ($Ly5.1^+$) → $CD44^{-/-}$ ($Ly5.1^+$). E, F. $CD44^{+/+}$ ($Ly5.1^+$ $Ly9.1^-$) & $CD44^{-/-}$ ($Ly5.1^+$ $Ly9.1^+$) → 1100rads $CD44^{+/+}$ ($Ly5.2^+$ Ly9.1). $CD4^+$ and $CD8^+$ thymocytes are gated on SP cells. The data are mean ± s.e.m, A-D from 2 groups of 8 chimeras, and E-F from 1 group of 6 chimeras. P values, 2-sample t test; ***, $P<0.005$, ****, $P<0.001$,

A significantly lower proportion of both $CD4^+$ and $CD8^+$ T cells was again found in the thymus compared to the spleen and the LN (Figures 5.31E & F). There was also a difference found between the spleen and the LN in both the $CD4^+$ and $CD8^+$ $CD44^{-/-}$ populations where there was a lower percentage of $CD44^{-/-}$ T cells found in the LN.

5.3.6.4. $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) and $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras

To eliminate any contribution of host T or B cells to the distribution of donor derived cells, chimeras were generated in $RAG2^{-/-}$ hosts as described in section 3.3.7. In this case, a significantly lower proportion of $CD4^+$ T cells but not $CD8^+$ T cells was found in the thymus compared to the spleen and the LN (Figures 5.32A & B).

5.3.6.5. HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras.

To eliminate the possibility that contaminating mature lymphocytes in the donor BM affected the distribution of $CD44^{-/-}$ cells in mixed chimeras, additional chimeras were generated using lineage depleted BM as described in section 3.3.9. As with the previous $RAG2^{-/-}$ chimeras, a significantly lower proportion of $CD4^+$ T cells but not $CD8^+$ T cells was found in the thymus compared to the spleen and the LN (Figures 5.32C & D).

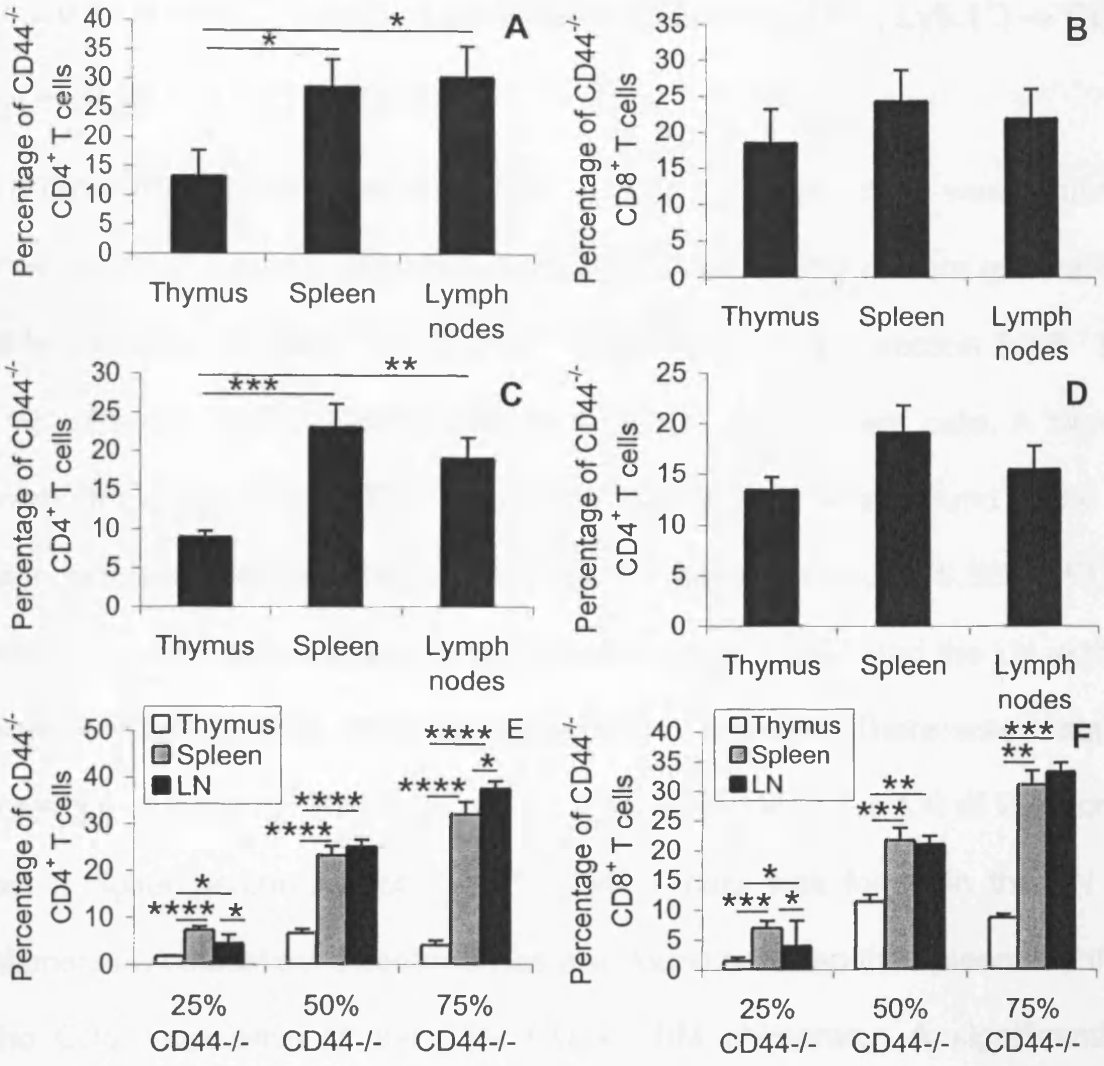

Figure 5.32. Analysis of the percentage of $CD4^+$ and $CD8^+$ $CD44^{-/-}$ cells in the thymus spleen and lymph nodes of various chimeras, II.

A, B. $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) and $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$). C, D. HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$). E, F. x$CD44^{+/+}$ & y$CD44^{-/-}$→ $CD44^{+/+}$. The data are mean ± s.e.m, A-D from 2 groups of 6 chimeras, and E-F from 3 group of 4 chimeras, each reconstituted with 25, 50, or 75% $CD44^{-/-}$ bone marrow. P values, 2-sample t test; *, P<0.05, **, P<0.01, ***, P<0.005, ****, P<0.001.

5.3.6.6. xCD44$^{+/+}$ (Ly9.1^{-}, Ly5.2^{+}) and yCD44$^{-/-}$ (Ly9.1^{+}, Ly5.1^{+}) → CD44$^{+/+}$ (Ly9.1^{-}, Ly5.1^{+}) chimeras.

To determine whether the distribution of CD44$^{-/-}$ lymphocytes was influenced by their relative frequency compared to CD44$^{+/+}$ cells, chimeras were generated using different ratios of CD44$^{-/-}$ and CD44$^{+/+}$ BM as described in section 3.3.8. Ly9 and Ly5 antibodies were used to distinguish donor and recipient cells. A significantly lower proportion of both CD4^{+} and CD8^{+} T cells was again found in the thymus compared to the spleen and the LN in all the chimeras (Figures 5.32E & F). There was also a statistical difference found between the spleen and the LN in the CD4^{+} population of the 25% and 75% CD44$^{-/-}$ BM chimeras. There was a statistically lower percentage of CD44$^{-/-}$ CD4^{+} T cells was found in the LN of 25% chimeras, but a higher percentage of CD44$^{-/-}$ CD4^{+} T cells was found in the LN of 75% chimeras. A statistical difference was also found between the spleen and the LN in the CD8^{+} population of the 25% CD44$^{-/-}$ BM chimeras. A significantly lower percentage of CD44$^{-/-}$ CD8^{+} T cells was found in the LN than the spleen.

5.3.7. Normal TCRvβ usage in periphery of CD44$^{+/+}$ and CD44$^{-/-}$ T cells.

The relatively poor contribution of CD44$^{-/-}$ cells to the thymocytes when in competition with CD44$^{+/+}$ cells raised the possibility that surviving CD44$^{-/-}$ cells might represent a selected subset, which could be linked to TCR specificity. Selection on the basis of TCR usage might also occur in the periphery, given that

the percentage of $CD44^{-/-}$ T cells is much higher in the spleen or LN than the thymus. Selective survival or expansion could potentially produce a more limited diversity of T cells with a narrowing of their TCRvβ usage. To address this possibility, $CD4^{+}$ and $CD8^{+}$ splenic $CD44^{+/+}$ and $CD44^{-/-}$ T cells from chimeras, generated in a RAG hosts, were stained with a panel of TCRvβ antibodies and their frequencies analysed (Figure 5.33). There was little difference in the TCRvb usage in the $CD4^{+}$ or $CD8^{+}$ $CD44^{-/-}$ cells compared to the $CD44^{+/+}$ cells. Although a couple of statistically significant differences were observed there were more $CD4^{+}$ and $CD8^{+}$ $CD44^{-/-}$ T cells using TCRvβ7 and more $CD4^{+}$ $CD44^{-/-}$ cells using TCRvβ12, there was an overall similar distribution showing normal TCRvβ usage.

5.4. A role for CD44 in B cell development

5.4.1. CD229 expression in B cell development

CD229 (Ly9) has been utilised earlier in this chapter as well as in the previous chapter to help to elucidate a role for CD44 in T cell development. To ensure that it was also suitable for use as a marker in B cell development, its expression was investigated together with CD2 on B cells at different developmental stages. Ly9.1 was found on all stages of B cell development in 129Sv mice but on none of the cells from C57Bl/6 mice (Figures 5.34).

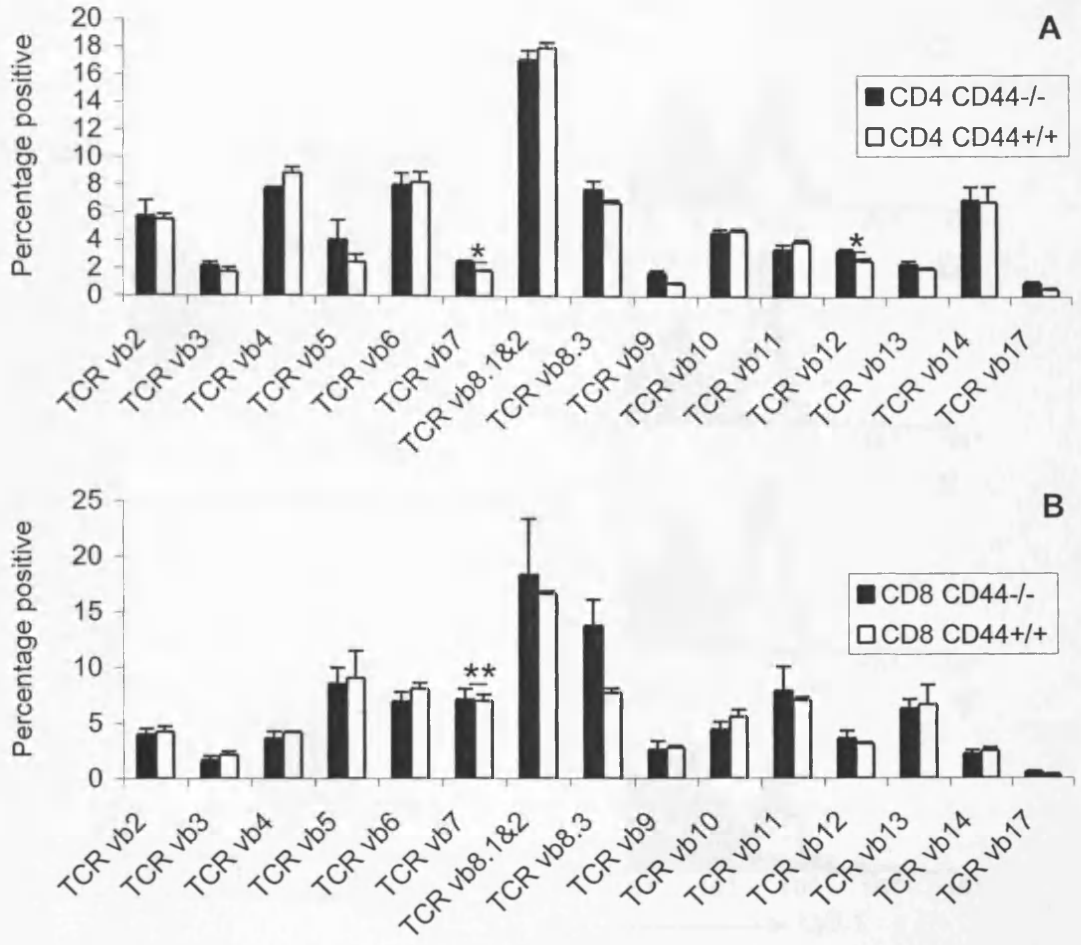

Figure 5.33. Analysis of the usage of vβ in $CD44^{-/-}$ vs. $CD44^{+/+}$ $CD4^+$ and $CD8^+$ T cells in the spleen of $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) and $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras.

A. Distribution of vβ usage in $CD44^{-/-}$ vs. $CD44^{+/+}$ $CD4^+$ T cells. B. Distribution of vβ usage in $CD44^{-/-}$ vs. $CD44^{+/+}$ $CD8^+$ T cells. The data are mean ± s.e.m, from 1 groups of 4 chimeras. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$.

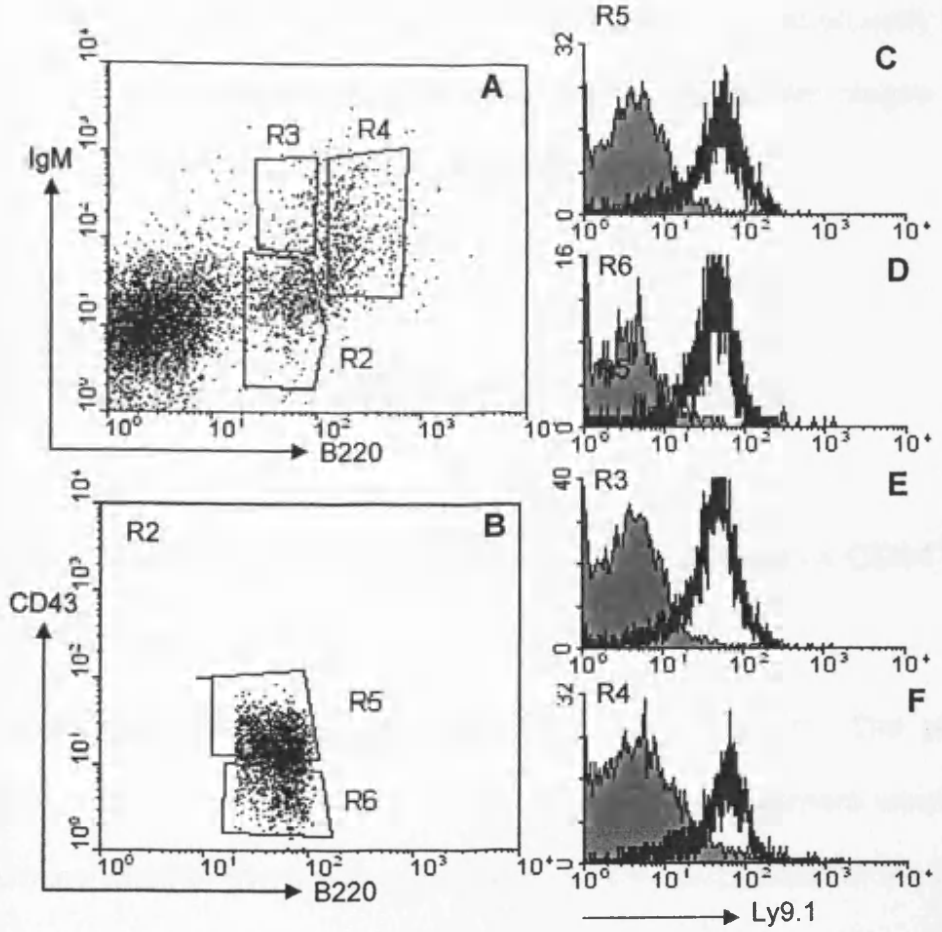

Figure 5.34. Analysis of the Ly9.1 expression in B cell development.

A. Definition of three groups of B cell development, by surface expression of IgM and B220. B. Definition of two early stages of B cell development by sIgM, CD43 and B220 staining gated on R2. C. Ly9.1 expression of R5. D. Ly9.1 expression of R6. E. Ly9.1 expression of R3. F. Ly9.1 expression of R4. Data are representative results from 2 129S/v mice, and 2 C57Bl/6 mice. C57Bl/6 mice■ 129S/v mice—

In contrast, CD2 was only expressed on a subset of cells at an early ($B220^{lo}$ $sIgM^-$) stage of B cell development, expressed on all cells at later stages (Figure 5.35). This finding was consistent in both strains of mice.

5.4.2. B cell development in mixed chimeras.

5.4.2.1. $CD44^{+/+}$ ($Ly5.2^+$) and $CD44^{-/-}$ ($Ly5.1^+$) donors → $CD44^{+/+}$ ($Ly5.2^+$) chimeras.

Chimeras were generated as described in section 3.3.1. The percentages of $CD44^{-/-}$ cells in the progressive stages of B cell development were compared to determine whether there was a difference in the composition of the developmental subsets. There was no statistical difference between the progressive stages, although there did appear to be a progressive increase in $CD44^{-/-}$ cells through the developmental stages (Figures 5.36A).

5.4.2.2. $CD44^{+/+}$ ($Ly5.2^+$) and $CD44^{-/-}$ ($Ly5.1^+$) donors → $CD44^{-/-}$ ($Ly5.1^+$) chimeras.

To determine whether CD44 expression by the host influenced B cell maturation, chimeras were generated in $CD44^{-/-}$ recipients as described in section 3.3.2. When the mixed BM chimeras were generated in $CD44^{-/-}$ hosts, there appeared to be significantly fewer $CD44^{-/-}$ cells in the $CD43^-$ $B220^{lo}$ $sIgM^-$ developmental stage than in the previous or following stage (Figures 5.36B).

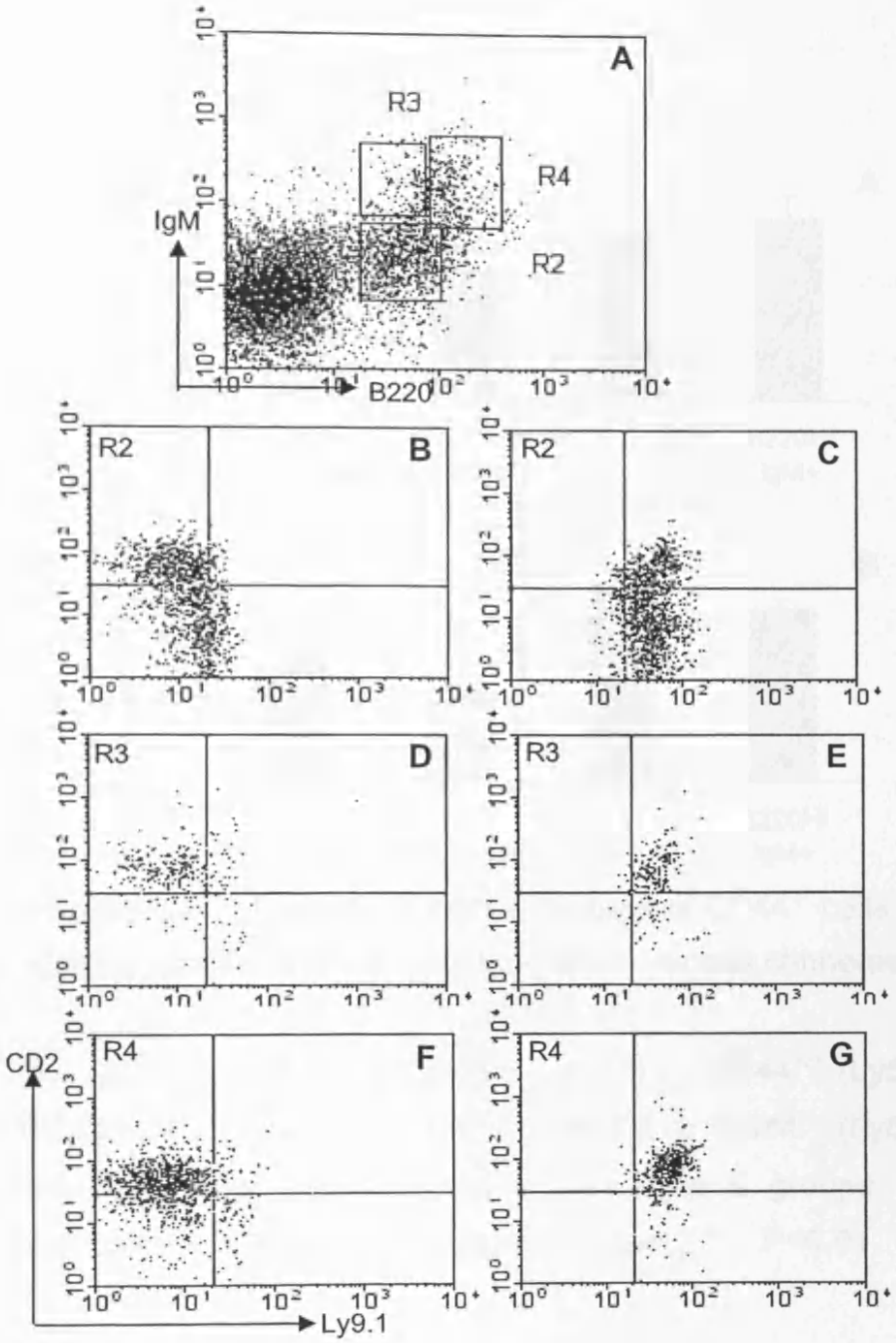

Figure 5.35. Distribution of Ly9.1 and CD2 on various B cells subsets.

A. B220 and sIgM expression in bone marrow cells defining 3 stages of B cell development. B, C. CD2 vs. Ly9.1 expression in R2. . D, E. CD2 vs. Ly9.1 expression in R3. F, G. CD2 vs. Ly9.1 expression in R4. A, B, D, F are C57Bl/6 mice and C, E, G, are 129S/v mice. Data are representative of results from 2 129S/v mice and 2 C57Bl/6 mice.

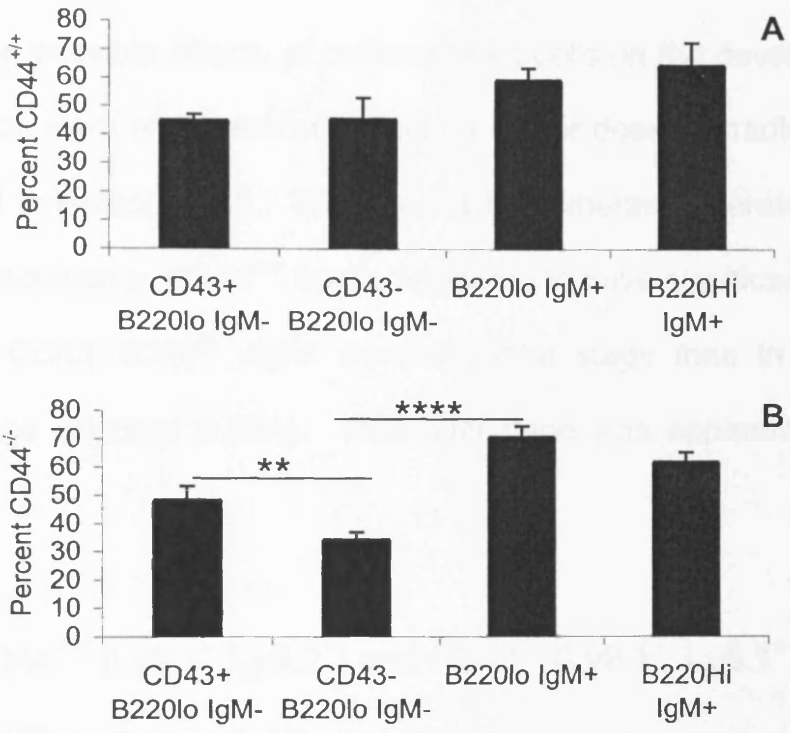

Figure 5.36. Analysis of the percentage of $CD44^{-/-}$ cells in various stages of B cell development in various chimeras, I.

A, $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly5.1^{+}$) $\rightarrow$ $CD44^{+/+}$ ($Ly5.2^{+}$).
B. $CD44^{+/+}$ ($Ly5.2^{+}$) & $CD44^{-/-}$ ($Ly5.1^{+}$) $\rightarrow$ $CD44^{-/-}$ ($Ly5.1^{+}$).
The data are mean ± s.e.m, A-D from 2 groups of 6 chimeras. P values, 2-sample t test; **, $P<0.01$, ****, $P<0.001$.

5.4.2.3. $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) and $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.1^+$) 1100 chimeras

To reduce the possible effects of radioresistant cells on the development of donor cells, chimeras were also generated using a higher dose of irradiation (1100 rads) as described in section 3.3.6. The mixed BM chimeras generated with a greater amount of irradiation in $CD44^{+/+}$ hosts, appeared to have significantly fewer $CD44^{-/-}$ cells in the $CD43^-$ $B220^{lo}$ $sIgM^-$ developmental stage than in the previous or following stage (Figures 5.37A). This phenotype was apparent in the previous chimeras.

5.4.2.4. $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) and $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras

To eliminate any contribution of host T or B cells to the distribution of donor derived cells, chimeras were generated in $RAG2^{-/-}$ hosts as described in section 3.3.7. There appeared to be little change in the different B cell developmental stages in these chimeras (Figures 5.38A).

5.4.2.5. HSC $CD44^{+/+}$ ($Ly9.1^-$, $Ly5.2^+$) & HSC $CD44^{-/-}$ ($Ly9.1^+$, $Ly5.1^+$) → $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^-$, $Ly5.1^+$) chimeras.

To eliminate the possibility that contaminating mature lymphocytes in the donor BM affected the distribution of $CD44^{-/-}$ cells in mixed chimeras, additional chimeras were generated using lineage depleted BM as described in section 3.3.9.

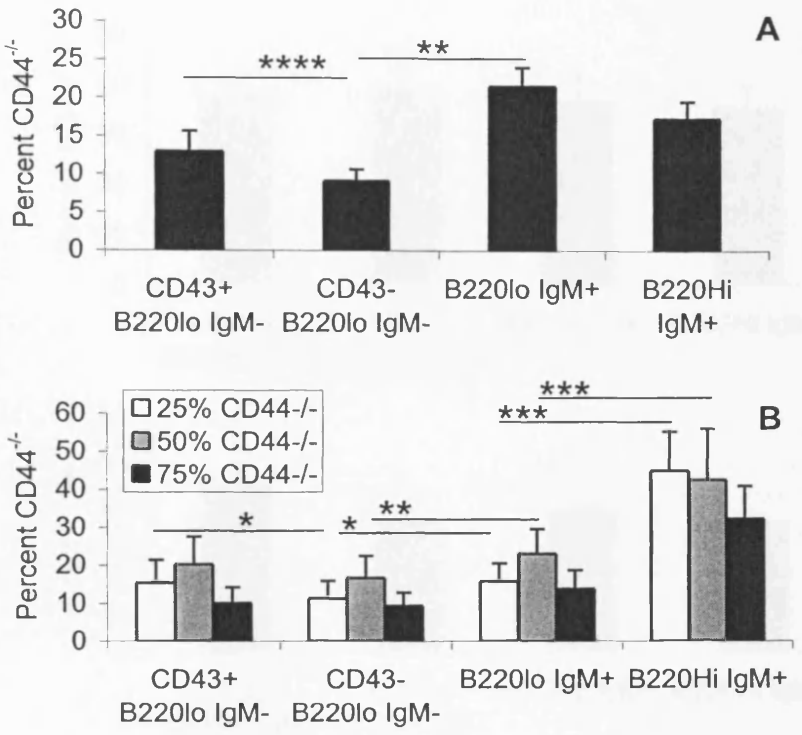

Figure 5.37. Analysis of the percentage of $CD44^{-/-}$ cells in various stages of B cell development in various chimeras, II.

A, $CD44^{+/+}$ ($Ly5.1^{+}$ $Ly9.1^{-}$) & $CD44^{-/-}$ ($Ly5.1^{+}$ $Ly9.1^{+}$) → 1100rads $CD44^{+/+}$ ($Ly5.2^{+}$ Ly9.1). b. $xCD44^{+/+}$ & $yCD44^{-/-}$→ $CD44^{+/+}$. The data are mean ± s.e.m. A from 1 group of 6 chimeras, and B from 3 groups of 6 chimeras, each reconstituted with 25, 50, or 75% $CD44^{-/-}$ bone marrow. P values, 2-sample t test; **, P<0.01, ****, P<0.001.

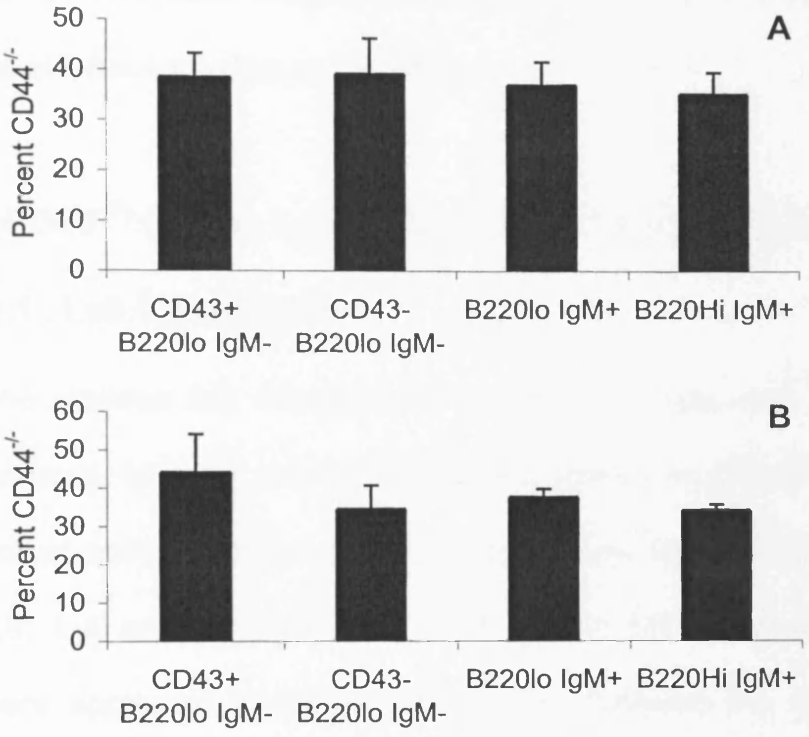

Figure 5.38. Analysis of the percentage of $CD44^{-/-}$ cells in various stages of B cell development in various chimeras, III.

A. $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) and $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) $\rightarrow$ $RAG2^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$). B. HSC $CD44^{+/+}$ ($Ly9.1^{-}$, $Ly5.2^{+}$) & HSC $CD44^{-/-}$ ($Ly9.1^{+}$, $Ly5.1^{+}$) $\rightarrow$ $RAG^{-/-}$ ($CD44^{+/+}$, $Ly9.1^{-}$, $Ly5.1^{+}$). The data are mean $\pm$ s.e.m, from 2 groups of 6 chimeras,

There were no statistical differences in the progressive B cell developmental stages in these chimeras (Figures 5.38B).

5.4.2.6. xCD44$^{+/+}$ (Ly9.1^{-}, Ly5.2^{+}) and yCD44$^{-/-}$ (Ly9.1^{+}, Ly5.1^{+}) → CD44$^{+/+}$ (Ly9.1^{-}, Ly5.1^{+}) chimeras.

To determine whether the development of CD44$^{-/-}$ B cells was influenced by the relative frequency of their precursor cells compared to CD44$^{+/+}$ cells, chimeras were generated using different ratios of CD44$^{-/-}$ and CD44$^{+/+}$ BM as described in section 3.3.8. Ly9 and Ly5 antibodies were used to distinguish donor and recipient cells. There appeared to be little correlation between the composition of the reconstitution inoculum and the percentage of CD44$^{-/-}$ cells found within the different developmental pathways (Figure 5.37B). However, it was evident that a greater proportion of CD44$^{-/-}$ cells was found in the most mature (B220^{+}IgM^{hi}) stage at all ratios of reconstitution.

5.4.3. B cell development in thymectomised mice

The role of CD44 in directing precursor cells to the thymus could, in an indirect way, impact on the production of B cells by CD44$^{-/-}$ cells in chimeric mice. Hence it is possible that CD44$^{-/-}$ precursors that fail to gain entry to the thymus could potentially migrate to the BM and generate B cells. To investigate whether this could occur, a group of chimeric mice were generated with mixed BM using either irradiated thymectomised or sham thymectomised mice as recipients.

If biased production of B cells by $CD44^{-/-}$ precursors in mixed chimeras results from preferential homing of $CD44^{+/+}$ precursors to the thymus, this bias should be eliminated in thymectomised mice.

$Ly5.1^{+}$ mice were thymectomised or sham thymectomised and allowed to recover for 7 weeks. Lineage depleted BM cells from $CD44^{+/+}$ ($Ly5.2^{+}$, $Ly9.1^{-}$) and $CD44^{-/-}$ ($Ly5.1^{+}$, $Ly9.1^{+}$) mice were injected in a 1:1 ratio into irradiated recipients. The mice were then left to reconstitute before their phenotype was analysed.

In sham-thymectomised recipients, normal percentages of total T and B cells were observed, (Figures 5.39A, 5.40A). As expected the thymectomised mice had few T cells and a higher percentage of B cells. Surprisingly, however, small numbers of donor T cells were present in thymectomised recipients (Figure 5.39B & 5.40B). This suggests either incomplete thymectomy in some recipients or incomplete T cell depletion from the donor bone marrow. Nevertheless, the key finding from this experiment was that the percentage of $CD44^{-/-}$ B cells was not reduced in thymectomised chimeras (Figure 5.39B & 5.40B). This suggests that failed circulation of precursors to the thymus does not contribute significantly to the biased B cell production of $CD44^{-/-}$ cells. Analysis of mature B cell subsets (Figures 5.41 and 5.42) and immature B cells in the bone marrow revealed no differences between thymectomised and sham thymectomised mice (Figure 5.43).

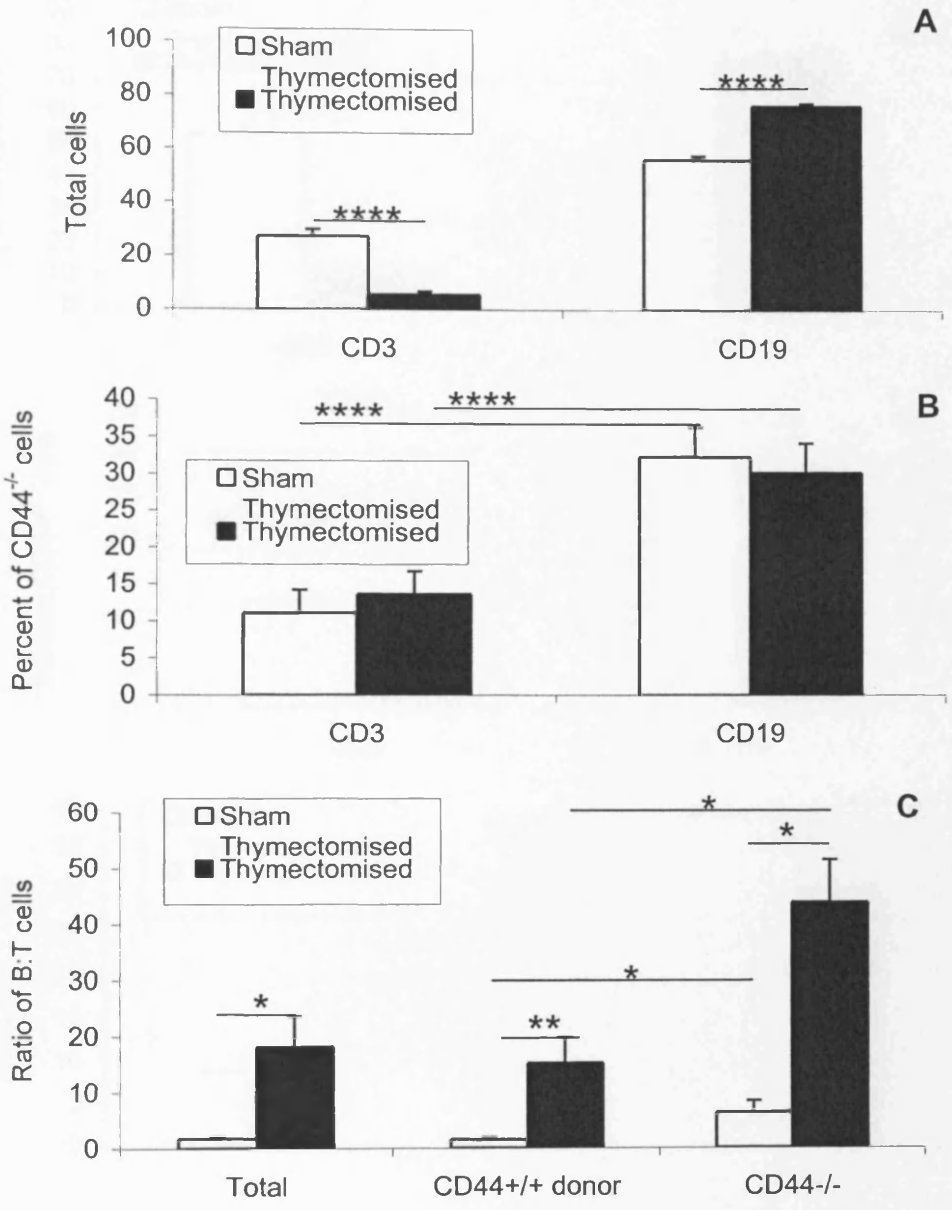

Figure 5.39. Analysis of T and B cells in the spleens of thymectomised and sham thymectomised chimeras.

A. Percentages of T and B cells in spleen of the different chimeras. B. The percentage of $CD44^{-/-}$ ($Ly9.1^{+}$) cells in each splenic population. C. The B:T cell ratio among total and $CD44^{-/-}$ and $CD44^{+/+}$ donor populations in the thymectomised and sham thymectomised chimeras. The data are mean ± s.e.m. obtained from 2 groups of 5 chimeras. P values, 2-sample t test ; *, $P<0.05$, **, $P<0.01$, ****, $P<0.001$.

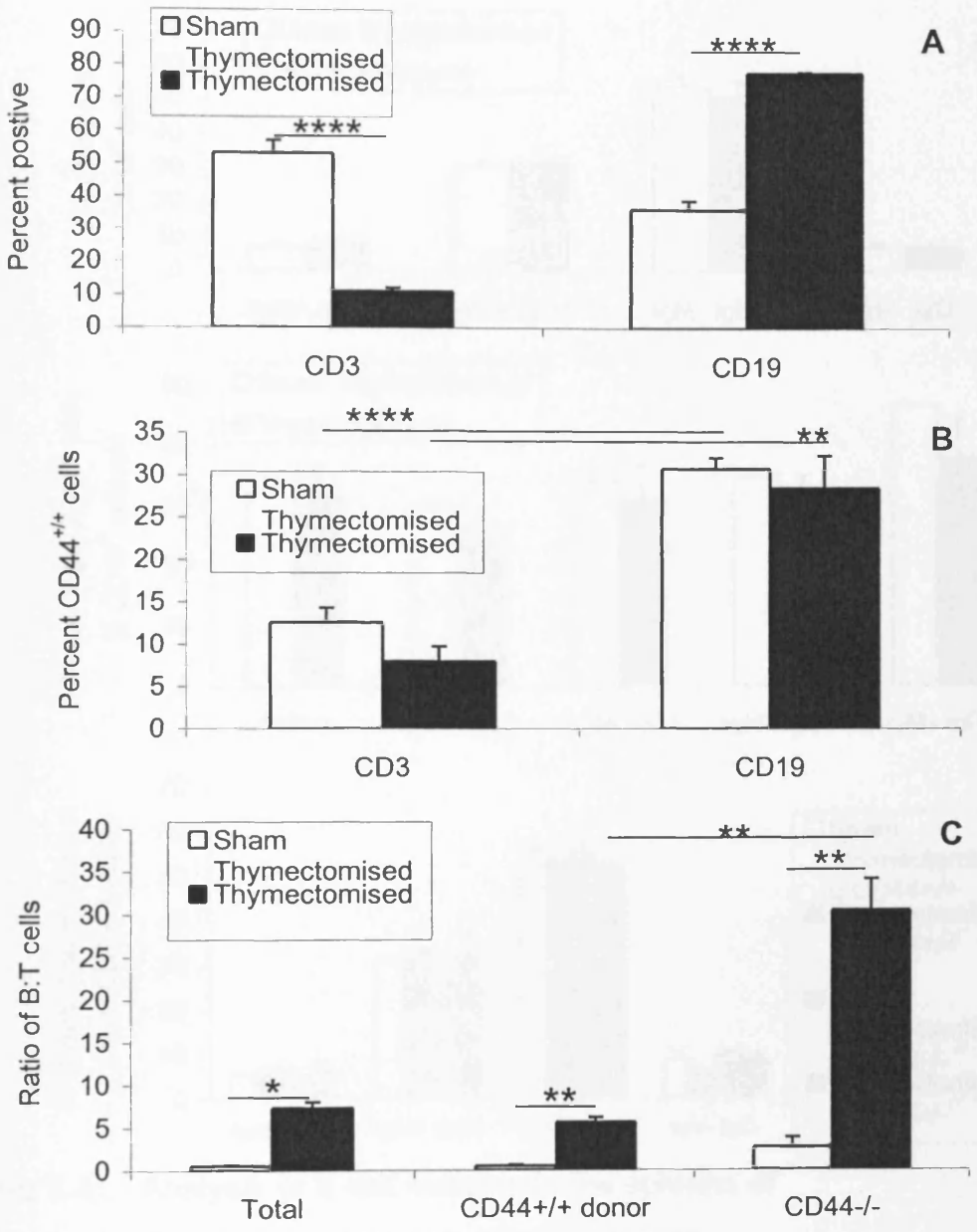

Figure 5.40. Analysis of T and B cells in lymph nodes of thymectomised and sham thymectomised chimeras.

A. Percentages of T and B cells in the lymph nodes of the different chimeras. B. The percentage of $CD44^{-/-}$ ($Ly9.1^{+}$) cells in each lymph node population. C. The B:T cell ratio among total and $CD44^{-/-}$ and $CD44^{+/+}$ donor populations in the thymectomised and sham thymectomised chimeras. The data are mean ± s.e.m. obtained from 2 groups of 5 chimeras. P values, 2-sample t test ; *, $P<0.05$, **, $P<0.01$, ****, $P<0.001$.

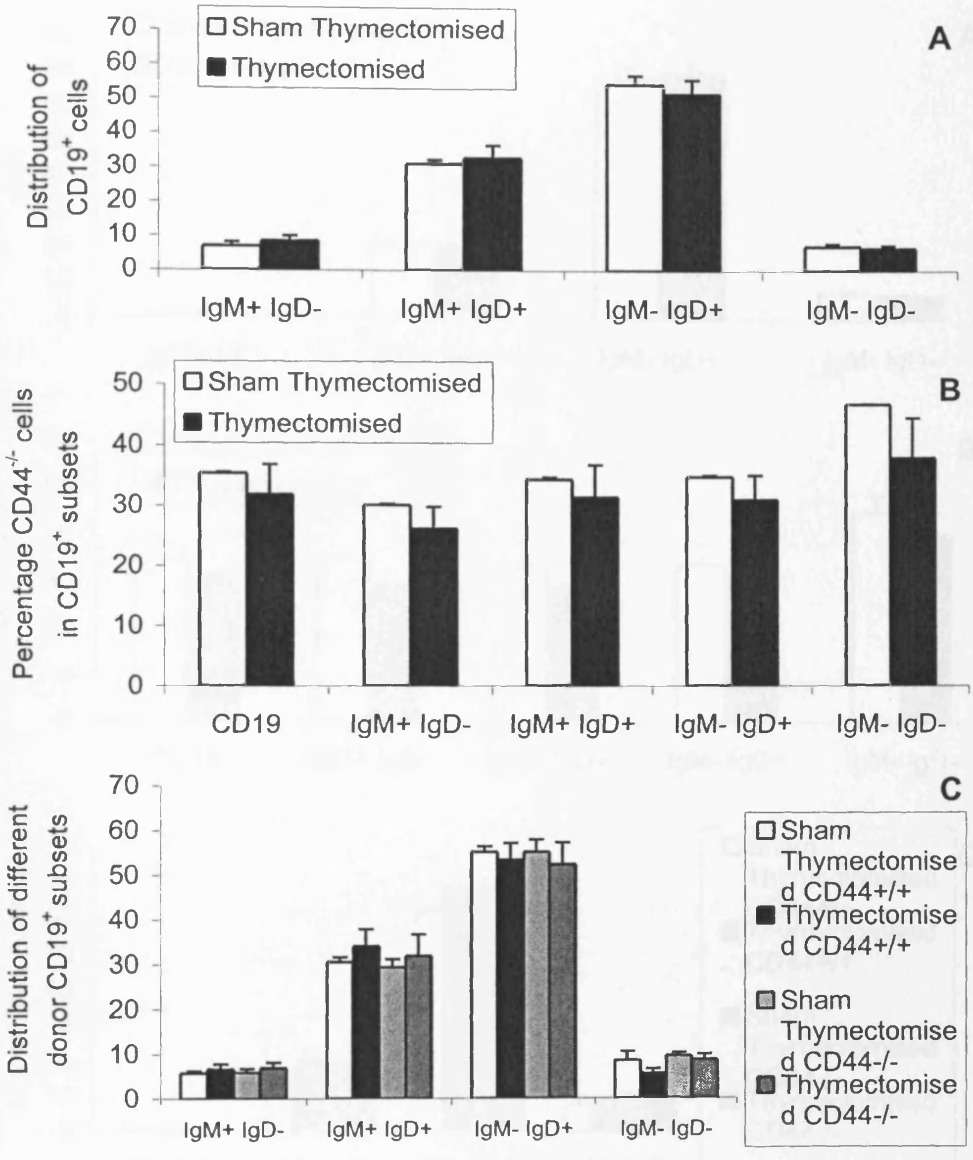

Figure 5.41. Analysis of B cell subsets in the spleens of thymectomised and sham thymectomised chimeras.

A. Percentages of total splenic B cell ($CD19^+$) subsets defined by sIgM and sIgD expression in different chimeras. B. The percentage of $CD44^{-/-}$ ($Ly9.1^+$) cells in each B cell subset. C. Distribution of sIgM and sIgD staining among $CD44^{-/-}$ or $CD44^{+/+}$ $CD19^+$ cells in thymectomised or sham thymectomised chimeras. Data are mean ± s.e.m. obtained from 2 group of 5.

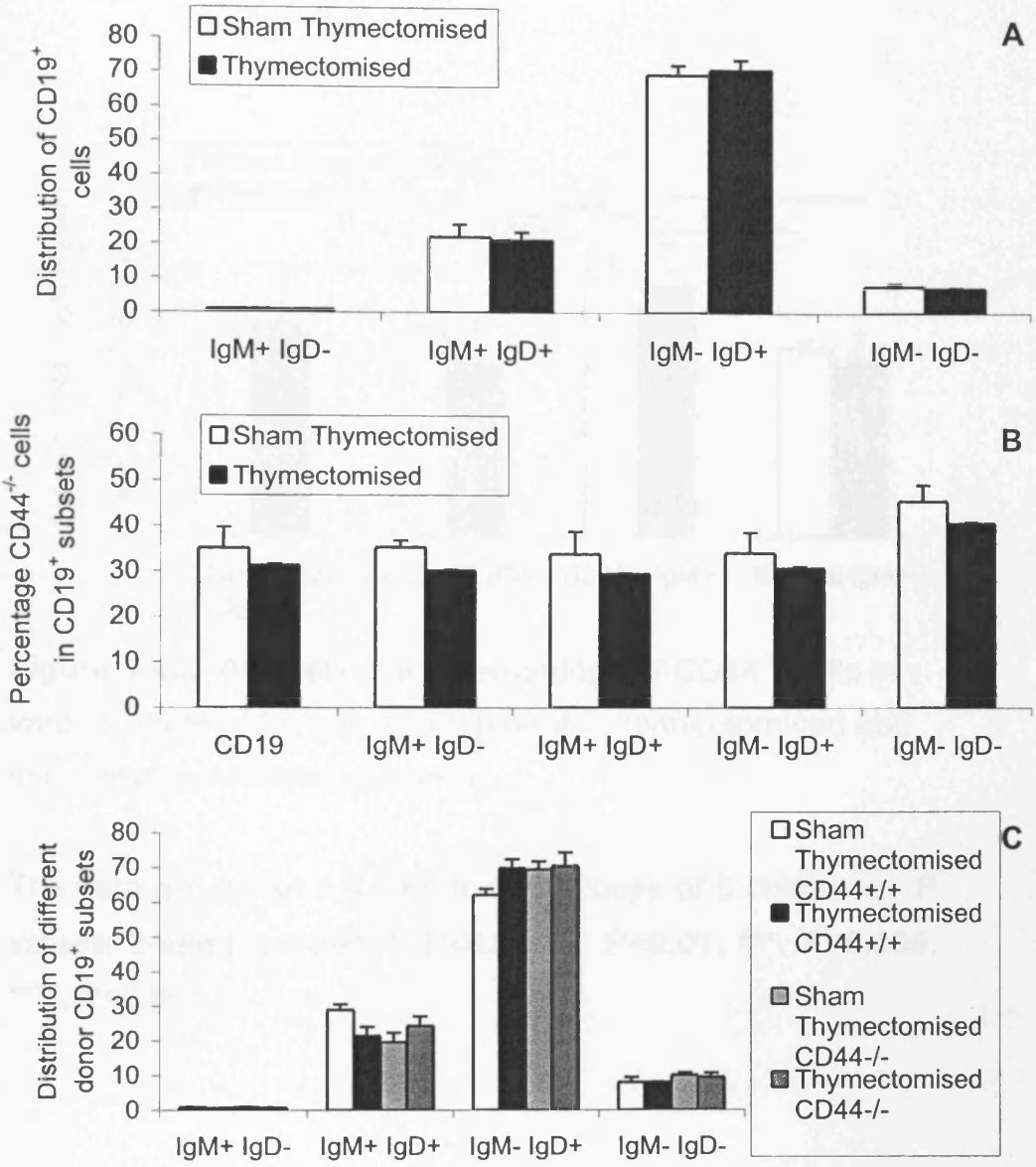

Figure 5.42. Analysis of B cell subsets in the lymph nodes of thymectomised and sham thymectomised chimeras.

A. Percentages of total B cell ($CD19^+$) subsets defined by sIgM and sIgD expression in the lymph nodes of the different chimeras. B. The percentage of $CD44^{-/-}$ ($Ly9.1^+$) cells in each B cell subset. C. Distribution of sIgM and sIgD staining among $CD44^{-/-}$ or $CD44^{+/+}$ $CD19^+$ cells in thymectomised or sham thymectomised chimeras. Data are mean ± s.e.m. obtained from 2 group of 5.

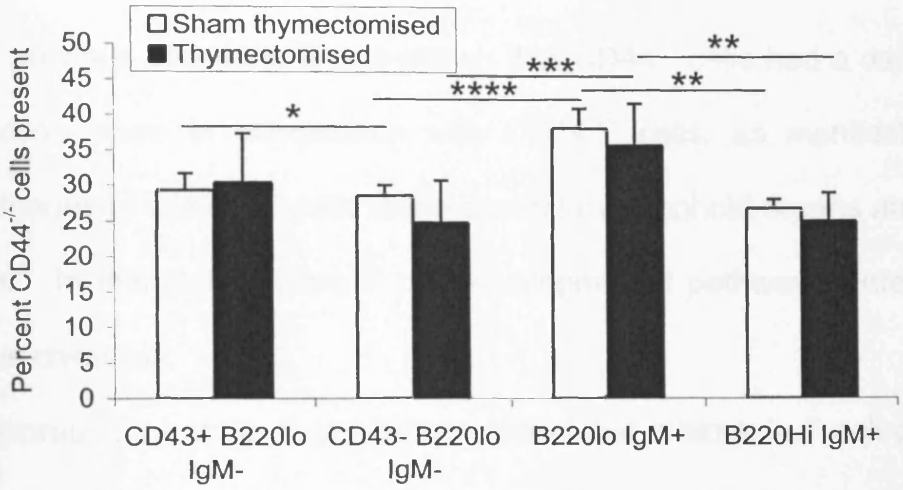

Figure 5.43. Analysis of the percentage of $CD44^{-/-}$ cells in various stages of B cell development in thymectomised and sham thymectomised chimeras.

The data are mean ± s.e.m, from 2 groups of 5 chimeras. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$.

5.5. Summary of T cell development

In the previous chapters, it was shown that $CD44^{-/-}$ cells had a deficiency in T cell production when in competition with $CD44^{+/+}$ cells, as manifested by reduced percentages of $CD44^{-/-}$ T cells in the secondary lymphoid organs and the peripheral tissues. In this chapter, the T cell developmental pathways were investigated in various chimeras.

In interpreting the data, it can be concluded that a block in T cell development will result in an increase in a particular thymocyte subset. In this respect, it is noteworthy that there was an increase in the percentage of $CD44^{-/-}$ cells in the DN stage of T cell development in all chimeras. There was also a relative increase in the proportion of $CD44^{-/-}$ cells at the SP stages and a reduction at the DP stage. These results suggest that there is reduced development from the DN to the DP stage among $CD44^{-/-}$ cells, but that the DP to SP transition is not reduced.

As the DN stage of T cell development can be divided into four populations, further investigations were carried out to determine whether $CD44^{-/-}$ cells were deficient in progressing through any particular DN stage. This analysis was more difficult to interpret, due to the very small percentages of cells in these subsets. However, the data appeared to show a decrease in the transition of $CD44^{-/-}$ cells from DN1 to DN2, an increase in the movement from DN2 to DN3, and a decrease in DN3 to DN4 maturation. Although this trend appeared in most the various mixed chimeras analysed, not all of the differences were statistically significant or consistent.

Perhaps surprisingly the analysis of chimeras four weeks after the intrathymic injection of progenitor cells demonstrated fewer $CD44^{-/-}$ cells in the DN subset than

the DP subset. This probably reflects limited renewal of progenitors injected into the thymus. As shown in the previous chapter, re-seeding of the thymus from BM progenitors still occurred after intrathymic injection, and this was evident in the greater percentage of $CD44^{-/-}$ cells among DN cells at 6 weeks post-injection. This phenomenon was also observed with respect to the DN subsets. Four weeks post-injection there was a higher percentage of $CD44^{-/-}$ cells among DN2 vs. DN1, and among DN4 vs. DN1 cells. By the following time point this trend had completely reversed to results comparable to *i.v.* injection, with DN1 having a higher percentage of $CD44^{-/-}$ cells than DN2 and DN4 a higher percentage than DN3. Overall, the main conclusion from the chimeras generated after *i.t.* injection was that the poor generation of T cells by $CD44^{-/-}$ progenitors in competition with $CD44^{+/+}$ cells was not due strictly to reduced thymic entry.

Whist, analysing the thymic data it was also of interest to note there was a markedly lower percentage of $CD44^{-/-}$ $CD4^{+}$ and $CD8^{+}$ cells in the thymus compared than in the spleen and LN. This could potentially be due to peripheral proliferation of $CD44^{-/-}$ cells, although why they should expand faster than CD44+/+ cells is unclear. In this regard, it has been reported that $CD44^{-/-}$ cells are more resistant to AICD (255). Since the $CD44^{-/-}$ cells have a normal TCRvβ usage, any peripheral expansion would need to be polyclonal in nature. In addition, this finding suggests that $CD44^{-/-}$ T cell development produces a full repertoire of $CD4^{+}$ and $CD8^{+}$ $CD44^{-/-}$ T cells.

5.6. Summary of B cell development.

The results of experiments investigating an influence of CD44 on B cell development were inconclusive. In two thirds of the chimeras there was a reduction of $CD44^{-/-}$ cells between the first ($CD43^{+}B220^{lo}sIgM^{-}$) and the second ($CD43^{-}B220^{lo}sIgM^{-}$) developmental stage. In five out of the six mixed chimeras there was an increase in $CD44^{-/-}$ cells from the second ($CD43^{-}B220^{lo}sIgM^{-}$) to the third stage ($B220^{lo}sIgM^{+}$) of B cell development. Finally in two thirds of the chimeras, a decrease in the percentage of $CD44^{-/-}$ cells was observed between the third ($B220^{lo}sIgM^{+}$) and fourth ($B220^{hi}sIgM^{+}$) stages of B cell development. Although differences were observed, not all of these were statistically significant. Overall, it appeared that there was an increase in $CD44^{-/-}$ cells at stages 1 and 3.

To determine whether production of $CD44^{-/-}$ B cells in mixed chimeras may have been boosted by precursors failing to gain entry to the thymus and returning to the BM chimeras were generated in thymectomised mice. In these mice, both $CD44^{+/+}$ and $CD44^{-/-}$ cells destined for the thymus could return to the BM and join the B cell development pathway. These results were complicated by the presence of some donor T cells in the thymectomised mice, possibly generated in remnants of the host thymus. Nevertheless, the percentage of T cells produced was greatly reduced indicating that thymic function was largely abrogated. Importantly, there was no difference in the percentage of $CD44^{-/-}$ B cells between the thymectomised and sham-thymectomised chimeras. Therefore, depletion of $CD44^{+/+}$ progenitors by homing to the thymus did not appear t play a role in the biased production of B cells by $CD44^{-/-}$ progenitors. It was also interesting to note that the $CD44^{-/-}$ cells in

the BM showed the same increases in stages 1 and 3 of B cell development as referred to above.

5.7.Conclusion

In conclusion it has been shown that there is an increase in $CD44^{-/-}$ cells present in the DN stage of thymic development, meaning that there is a deficiency in the ability of $CD44^{-/-}$ cells to progress from the DN to DP stage. An accumulation of $CD44^{-/-}$ cells at the DN stages 1 and 3 was also suggested, although the evidence was less clear. The relative decrease among $CD44^{-/-}$ thymocytes appeared to be greater for the DP subset than for SP cells. There could be several possible explanations. A, $CD44^{-/-}$ DP cells may transit more rapidly than $CD44^{+/+}$ DP cells to the SP stages. B, $CD44^{-/-}$ DP cells not selected for progression may die more rapidly than similarly unselected $CD44^{+/+}$ DP cells. C, $CD44^{-/-}$ SP cells may persist longer in the thymus than CD44SP cells, perhaps due to a deficiency in entering circulation. The latter explanation seems unlikely since a consistently higher percentage of $CD44^{-/-}$ cells was observed among SP cells in the thymus. It may be of interest to examine the turnover of thymocytes subsets in mixed chimeras to investigate further the reasons for the altered subset composition.

Two conclusions can be made with respect to the role of CD44 in B cell development. First failed homing of cells intended to become T cells does not promote increased production of $CD44^{-/-}$ B cells. Second, $CD44^{-/-}$ B cells appear to have an altered efficiency of differentiation that leads to an increase in the

proportion of cells at developmental stages 1 and 3. The reasons for these differences are presently unknown.

6. Chapter 6: Functional analysis of $CD44^{-/-}$ cells when placed in competition with $CD44^{+/+}$ cells

6.1. Introduction

In the previous sections we have demonstrated that CD44 has a probable role in lymphocyte development apart from thymic and BM entry. In this next section we will investigate whether the $CD44^{-/-}$ cells have any functional deficiencies when compared to $CD44^{+/+}$ cells. Some investigation has been carried out by other groups using $CD44^{-/-}$ mice and comparing them to $CD44^{+/+}$ mice. In addition, the role of CD44 has been studied using blocking antibodies and with transgenic cells that express only one variant exon. Both *in vivo* and *in vitro* experiments have been performed to identify any deficiencies or advantages that the $CD44^{-/-}$ cells may have. In one example, $CD44^{-/-}$ NK cells were shown to be deficient in binding to and killing certain target cells (177). In another, the presence of v4-7 CD44 on the T cell surface was shown to increase the T cell response to mitogens (179). Variant isoforms of CD44 have been implicated in DC maturation and function. Human blood derived DCs upregulate v3, v6 &v9 after TNF-α stimulation, and anti-variant CD44 antibodies subsequently induce DC aggregation, the upregulation of accessory molecules and the secretion of cytokines (117). Furthermore, v4, v5, v6, and v9 are upregulated by human Langerhan's cells during migration. Blockage of these exons by antibodies *in vivo* prevented Langerhan's cell migration from the skin and binding to the T cell zones of the LNs (256). The same antibodies also severely inhibited induction of a delayed type hypersensitivity reaction *in vivo* (257).

CD44 has been implicated in cellular infiltration into inflammatory tissue, as treating mice with anti-CD44 antibodies was shown to decrease inflammation. However, whether anti-CD44 antibodies acted by preventing infiltration or by triggering apoptosis is unclear (241). $CD44^{-/-}$ mice have been shown to recover slowly after experimental induction of lung inflammation. It was proposed that this may be due to the presence of excess extracellular matrix proteins, such as HA, which continually triggered an inflammatory response combined with a reduced ability of $CD44^{-/-}$ neutrophils to undergo apoptosis or to migrate out of the inflammatory site (258). This observation was repeated in tuberculosis infected mice, where there was an increased number of $CD44^{-/-}$ neutrophils present in the inflammatory site, demonstrating that CD44 was necessary for the resolution of the inflammatory response.

CD44 has also been linked to the production of cytokines. $CD44^{-/-}$ cells were also found to be deficient in TGF-β production in the context of an inflammatory response (139). In addition, CD44 has been linked to IFN-γ production. *toxoplasma gondii* infected mice had reduced IFN-γ production when anti-CD44 antibody was administered (258-260).

$CD44^{-/-}$ mice have been shown to have an elevated *in vivo* primary and secondary response, to conalbumin and staphylococcal enterotoxin A, which has been linked to $CD44^{-/-}$ cells being more resistant to activation induced cell death (AICD). The resistance to AICD was demonstrated for TCR-mediated apoptosis of thymocytes, which was not dependent on FAS. This group also demonstrated that injection of a blocking anti-CD44 antibody decreased the number of antigen specific T cells present during an immune response (255). In contrast, it has been reported that

$CD44^{-/-}$ epithelial cells are more susceptible to apoptosis, (261). In addition, CD44 deficient mice were found to exhibit enhanced hepatitis after ConA injection. In this experiment the same amount of ConA was given to both wild type and knock out mice, while the result being that the knock out mice died of liver damage; $CD44^{-/-}$ mice had increased levels of TNF-α, IL-2 and IFN-γ. Finally, it was also shown that T cells from $CD44^{-/-}$ mice were more resistant to activation induced cell death (262).

A role for CD44v7 in DTH responses has been suggested by studies showing that treatment with anti-CD44v7 could cure an inflammatory DTH response (180). It was proposed that the v7 exon provides survival signals and prevents the cell from undergoing apoptosis (183). CD44v10 has also been demonstrated to be involved in DTH (263). These data suggest that binding CD44 triggers survival and prevents apoptosis. This is in apparent contrast to the previously cited data suggesting the $CD44^{-/-}$ cells were more resistant to AICD.

Further contradictory information on whether CD44 prevents or promotes apoptosis has come from studies of Fas mediated apoptosis. Stimulation of CD44 was shown to decrease FAS expression and therefore FAS mediated apoptosis on lung cancer cells (260). However, another study demonstrated that signalling through CD44 up-regulates FAS on rheumatoid synovial cells (264).

In summary, there are a number of observations that suggest that CD44 is an important functional molecule on T cells, and that its properties are altered by expression of variant exons and the type of cell that is involved. However, the precise expression pattern of CD44 isoforms on T cell subsets at different stages

of activation and in different anatomical locations is not known. Furthermore, the importance of CD44 in immune functions overall remains unclear.

6.2.Aim.

To investigate the responses of $CD44^{-/-}$ T and B cells, to various immunological challenges when in competition with $CD44^{+/+}$ cells.

6.3.Functional DCs responses

Preliminary investigations were carried out on splenic DCs from $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras mixed chimeras from section 3.3.1. It was also attempted to analyse BMDCs from these chimeras, but due to membrane swapping in culture, it was difficult to differentiate between $CD44^{+/+}$ and $CD44^{-/-}$ BMDCs. Cell-cell interaction can cause surface molecules to move from one cell to another, when BMDCs were generated from chimeric mice it was very difficult to distinguish the two populations based on $Ly5.1^{+}$ staining (265). Therefore BM from either $CD44^{-/-}$ or $CD44^{+/+}$ mice was used to generate BMDCs.

6.3.1.1. Phenotype of splenic DCs from $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras

The spleen from the chimeras was digested using Dnase1 and Collagenase type IV, before the low density cells were collected by density centrifugation on

nycodenz (Figure 6.1A). The phenotype of the DC enriched fraction was analysed for subset composition and enrichment (6.1B & C).

There were statistically more $CD44^{-/-}$ DCs present in the enriched sample than the pre-enriched sample, this could indicate that the $CD44^{-/-}$ DCs contained more low-density cells than the $CD44^{+/+}$ cells. There also appeared to be statistical differences in the subset contribution of the DCs. There were more $CD8^{+}$ and $CD4^{+}$ DCs but fewer DN DCs derived from $CD44^{-/-}$ progenitors than in the $CD44^{+/+}$ DC population.

6.3.1.2. $CD44^{-/-}$ splenic DCs express a more activated phenotype.

A profile of the activation markers on $CD44^{+/+}$ and $CD44^{-/-}$ $CD11c^{+}$ cells were compared using the enriched splenic DCs from $CD44^{+/+}$ & $CD44^{-/-}\rightarrow$ $CD44^{+/+}$ chimeras. The $CD44^{-/-}$ DCs expressed significantly higher levels of the co-stimulatory molecules CD80, CD86 and CD40 than $CD44^{+/+}$ $CD11c^{+}$ cells, as measured by MFI (Figure 6.2A). They also had higher expression of the adhesion molecule ICAM-1. This was associated with a higher percentage of $CD44^{-/-}$ $CD11c^{+}$ showing positive expression of these markers (Figure 6.2B). In addition, ICAM-1 expression levels were the higher of ICAM-1^{+} $CD44^{-/-}$ than $CD44^{+/+}$ CD11c (Figure 6.2C).

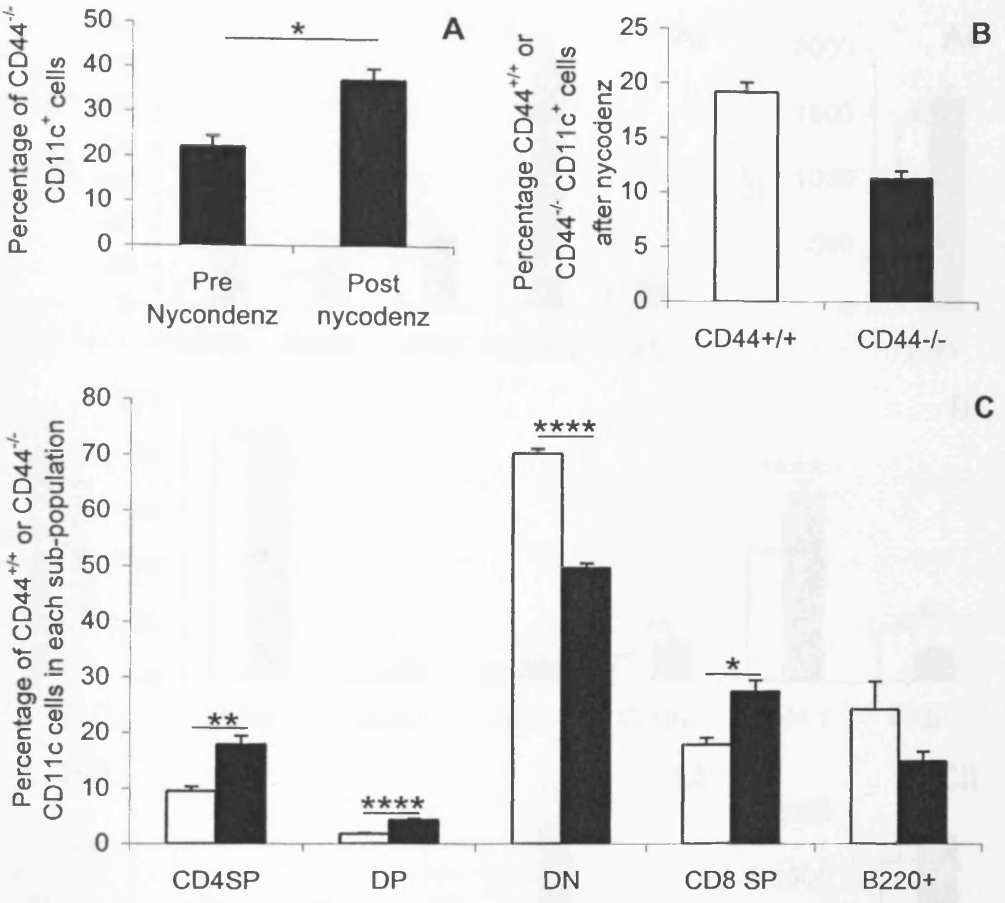

Figure 6.1. CD11c^{+} cells in spleen of CD44$^{+/+}$ & CD44$^{-/-}$→ CD44$^{+/+}$ chimeras.

A Percentage of CD44$^{-/-}$ CD11c splenic cells pre and post nycodenz enrichment. B, Purification of CD44$^{+/+}$ and CD44$^{-/-}$ CD11c cells after nycodenz enrichment. C, Phenotypic distribution of CD44$^{+/+}$ and CD44$^{-/-}$ CD11c splenic cells after nycodenz enrichment. The data are mean ± s.e.m. from 1 group of 4 chimeras. P values, 2-sample t test; *, P<0.05, **, P<0.01, ****, P<0.001. Black bars are CD44$^{-/-}$ cells (Ly5.1^{+}), and white bars are CD44$^{+/+}$ (Ly5.2^{+}) cells.

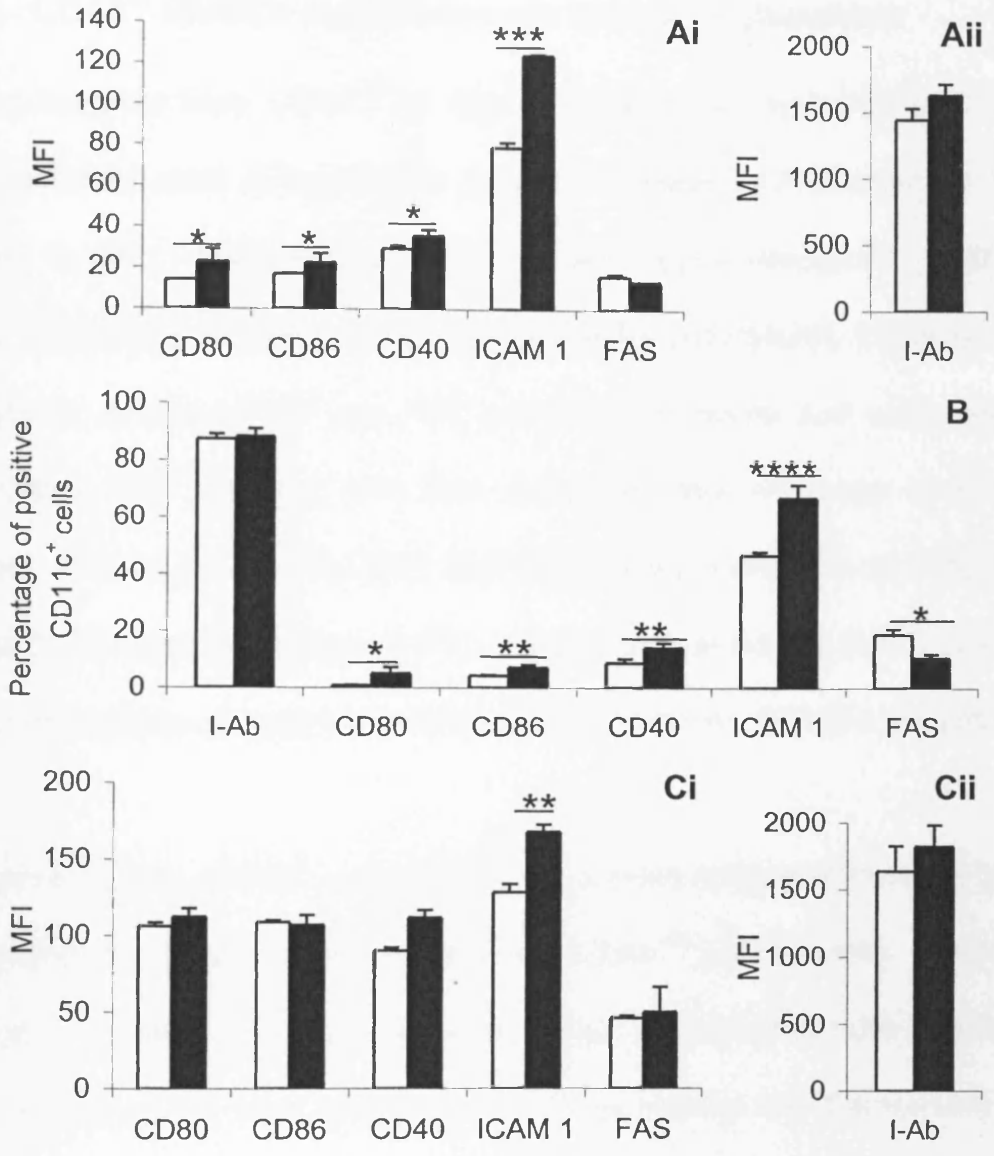

Figure 6.2. Expression of activation markers present on enriched DC's from spleens of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A Expression (MFI) of activation markers on $CD11c^+$ cells from $CD44^{+/+}$ or $CD44^{-/-}$ cells. B, Percent CD11c cells with positive expression of co-stimulatory molecules, $I\text{-}A^b$ is percentage of cells with high expression. C. MFI of $CD44^{+/+}$ or $CD44^{-/-}$ $CD11c^+$ cells, gated on positive expression, or $I\text{-}A^b$ high expression. The data are mean ± s.e.m. from a group of 4 chimeras. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, $P<0.005$, ****, $P<0.001$. Black bars are $CD44^{-/-}$ cells ($Ly5.1^+$), and white bars are $CD44^{+/+}$ ($Ly5.2^+$) cells.

6.3.1.3. $CD44^{-/-}$ BMDCs express a more immature phenotype

BMDCs generated from $CD44^{+/+}$ or $CD44^{-/-}$ mice were incubated at 37°C and at 4°C with different sizes of fluorescent particles to measure their ability to internalise molecules *in vitro*. Different particle sizes were used measure the efficiency of different endocytic pathways: a fluorescent dye for pinocytosis, the latex beads for phagocytosis, and the FITC and APC particles for macro and micro endocytosis respectively. The resultant MFI then measures the efficiency of the different pathways. This is equal to the MFI after 30 minutes incubation at 37°C minus the MFI after 30 minutes incubation at 4°C. Initial results indicated that $CD44^{-/-}$ $CD11c^{+}$ BMDCs cells uptake antigen better than $CD44^{+/+}$ $CD11c^{+}$ BMDCs (Figure 6.3A).

Among splenic DCs, $CD44^{-/-}$ cells expressed a more mature phenotype by virtue of higher expression of activation markers than $CD44^{+/+}$ CD11c cells. Since activated DC has a lower ability to uptake antigen, it was surprising to note that the $CD44^{-/-}$ BMDCs appeared to have a greater ability to uptake the fluorescent particles. However when the expression of the activation markers in the BMDCs were examined, the $CD44^{-/-}$ cells appeared to be more immature (Figure 6.3B). This was consistent for all activation markers except ICAM-1, which had a greater expression on the $CD44^{-/-}$ BMDCs.

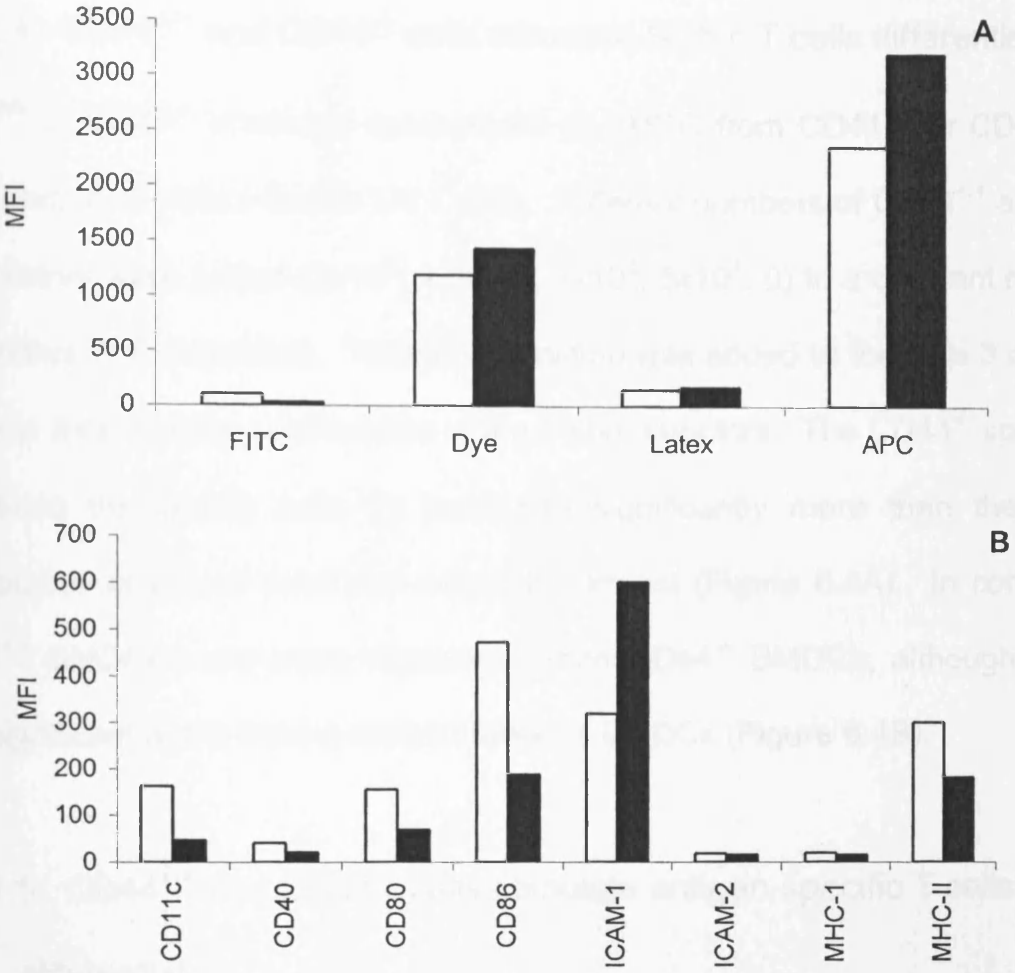

Figure 6.3. Expression of activation markers and uptake efficiency of $CD44^{+/+}$ and $CD44^{-/-}$ BMDC's.

A, Resultant MFI of $CD11c^{+}$ cells after 30-minute incubation with different sizes of fluorescent particles. B, Expression of activation markers on $CD44^{+/+}$ and $CD44^{-/-}$ BMDC's.
The data are from pooled BMDC's from 3 $CD44^{+/+}$ and 3 $CD44^{-/-}$ mice.
Black bars are $CD44^{-/-}$ BMDC's and white bars are $CD44^{-/-}$BMDC's.

6.3.1.4. $CD44^{+/+}$ and $CD44^{-/-}$ cells stimulate Balb/c T cells differentially.

$CD44^{+/+}$ or $CD44^{-/-}$ irradiated splenocytes or BMDC from $CD44^{+/+}$ or $CD44^{-/-}$ mice were used to stimulate Balb/c LN T cells. Different numbers of $CD44^{+/+}$ and $CD44^{-/-}$ stimulators were added ($2x10^5$, $1.5x10^5$, $1x10^5$, $5x10^4$, 0) to a constant number of responders (10^5 cells/well). Tritiated thymidine was added to the cells 3 days after set up to measure the proliferation of the Balb/c effectors. The $CD44^{-/-}$ splenocytes stimulated the Balb/c cells to proliferate significantly more than the $CD44^{+/+}$ splenocytes at all cell numbers except the lowest (Figure 6.4A). In contrast, the $CD44^{+/+}$ BMDCs were more stimulatory than $CD44^{-/-}$ BMDCs, although this was only significant at the lowest concentration of BMDCs (Figure 6.4B).

6.3.1.5. $CD44^{+/+}$ and $CD44^{-/-}$ cells stimulate antigen-specific T cells differentially.

BMDC were derived from either $CD44^{+/+}$ or $CD44^{-/-}$ mice. These were used to stimulate antigen specific transgenic $CD4^+$ or $CD8^+$ T cells. $CD4^+$ T cells were obtained from OT2 mice and are specific for a peptide in ovalbumin (266). $CD8^+$ T cells were obtained from 2C mice and are specific for L^d and also a K^b- restricted peptide (267). Spleens and LN were removed and pooled from the transgenic mice, B cell and MHCII depleted. Stimulation of $CD4^+$ T cells from the OT2 mice was assessed after pulsing BMDCs with different concentrations of ovalbumin protein. The $CD44^{+/+}$ BMDCs stimulated the T cells to proliferate more at higher concentrations of protein, whilst the $CD44^{-/-}$ cells appeared to stimulate the T cells better at lower concentrations.

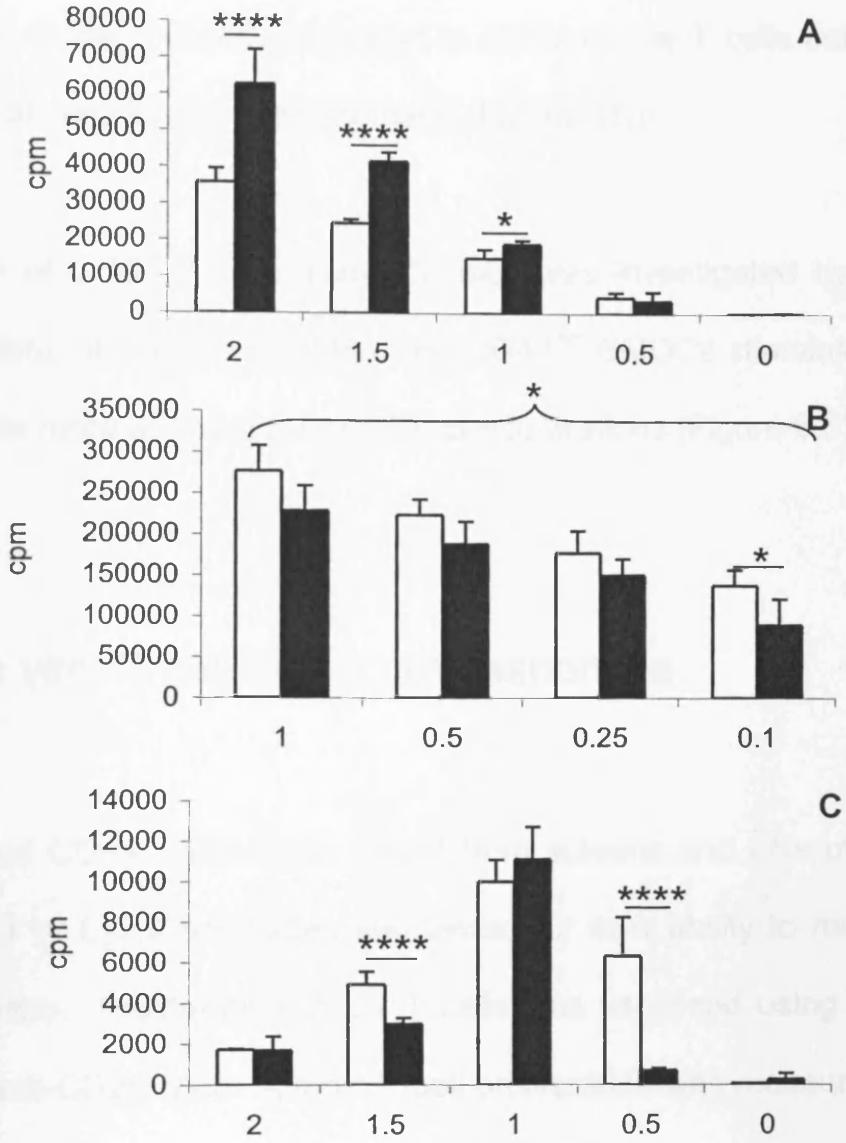

Figure 6.4. Non-specific stimulation (MLR) of $CD44^{+/+}$ $CD44^{-/-}$ and Balb/C effectors.

A, Balb/C effectors (LN cells) stimulated by different numbers of $CD44^{+/+}$ and $CD44^{-/-}$ irradiated splenocytes. B, Balb/C effectors (LN cells) stimulated by different numbers of $CD44^{+/+}$ and $CD44^{-/-}$ BMDC's. C, $CD44^{+/+}$ or $CD44^{-/-}$ effectors (LN cells) stimulated with different numbers of irradiated Balb/C splenocytes. The MLRs were carried out using 10^5 effectors cells per well, but a varied number of irradiated stimulators ranging from $2x10^5$ to $5x10^4$ in A and C, and $1x10^4$ to $1x10^3$ in B. The data are mean ± s.e.m. from 4 identical wells, using cells pooled from 3 mice, per strain. P values, 2-sample t test; *, $P<0.05$, ****, $P<0.001$.

Overall, the $CD44^{+/+}$ BMDC appeared to stimulate the T cells better (assessed by comparing all data in one statistical test) (Figure 6.5).

Stimulation of $CD8^{+}$ T cells from 2C mice was investigated by adding different concentrations of specific peptide. The $CD44^{+/+}$ BMDCs stimulated the 2C T cells to proliferate more at nearly all peptide concentrations (Figure 6.6).

6.4. *In vitro* T cell and B cell responses.

$CD44^{+/+}$ and $CD44^{-/-}$ cells were sorted from spleens and LNs of mixed chimeras using Ly5.1 or Ly5.2 antibodies and tested for their ability to respond to different stimuli *in vitro*. Proliferation of LN T cells was assessed using immobilised anti-CD3 and anti-CD28, while splenic B cell proliferation was measured in response to immobilised anti-IgM. There was very little difference between the $CD44^{+/+}$ and $CD44^{-/-}$ cells with respect to the anti-IgM-induced proliferative responses (Figure 6.7A). $CD44^{+/+}$ cells appeared to be more proliferative in the first two days, but the $CD44^{-/-}$ cells appeared to have a higher proliferation rate on days 4 to 5. This trend was also observed for the T cell response to anti-CD3 and anti-CD28 (Figure 6.7B). During days 2-3, $CD44^{+/+}$ cells appeared to be proliferating slightly more, although this difference was not statistically significant. However by days 4-5, $CD44^{-/-}$ cells had a statistically higher proliferative rate.

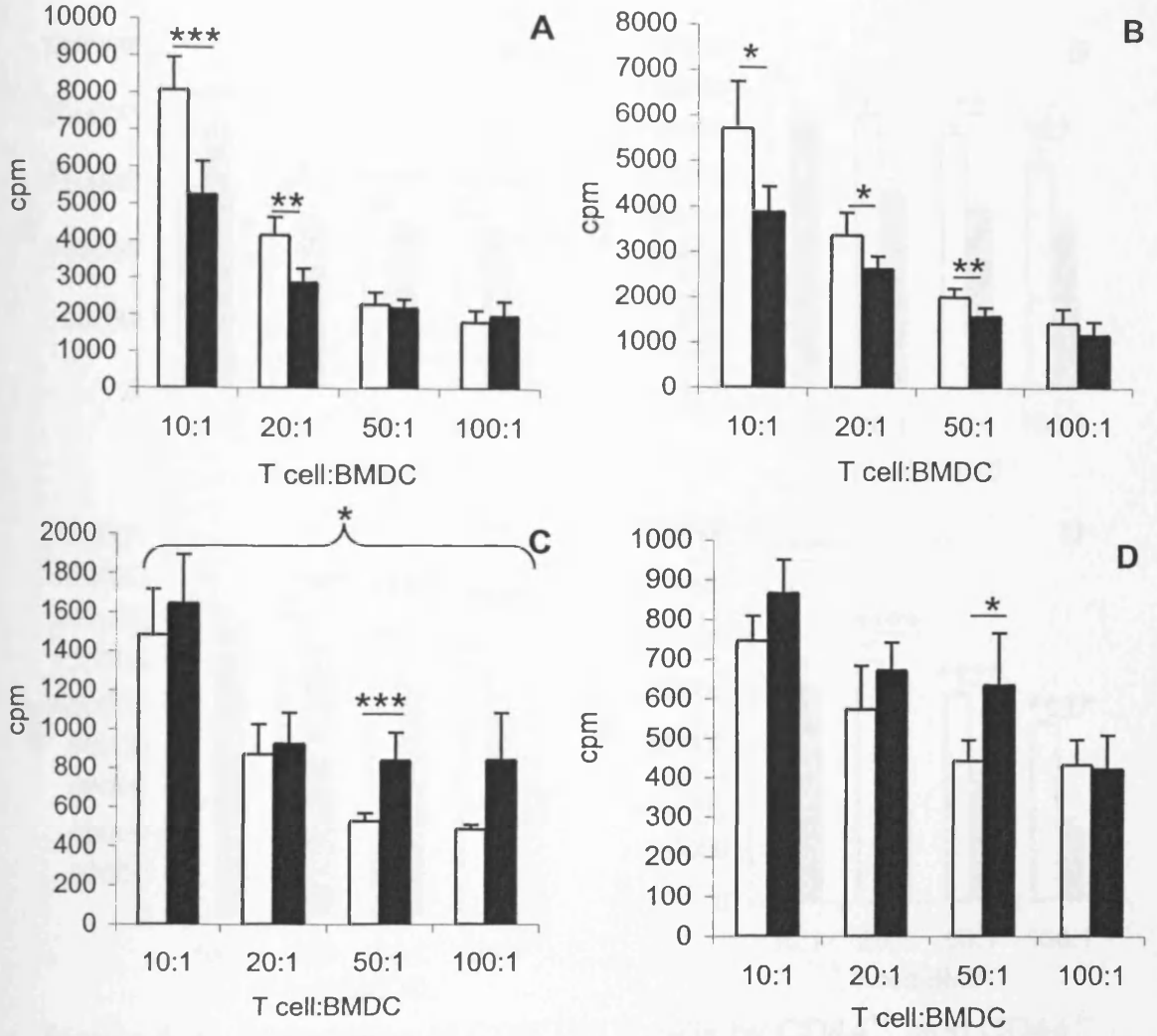

Figure 6.5. $CD44^{+/+}$ and $CD44^{-/-}$ specific stimulation of $CD4^{+}$ OT2 T cells.

$CD44^{+/+}$ and $CD44^{-/-}$ BMDC's were incubated with a varying amount of ova protein before being added to OT2 cells at various ratios. A, stimulators were incubated with 50μg of protein. B, stimulators were incubated with 25μg of protein. C, stimulators were incubated with 5μg of protein. D, stimulators were incubated with 1μg of protein. The data are mean ± s.d.m from 4 identical wells, using cells pooled from 3 mice. P values, 2-sample t test; *, $P<0.05$, ****, $P<0.001$. The black bars are $CD44^{-/-}$ and the white bars are $CD44^{+/+}$. Overall $CD44^{+/+}$ BMDCs are statistically better at stimulating OT2 cells than $CD44^{-/-}$ BMDCs. $P<0.005$.

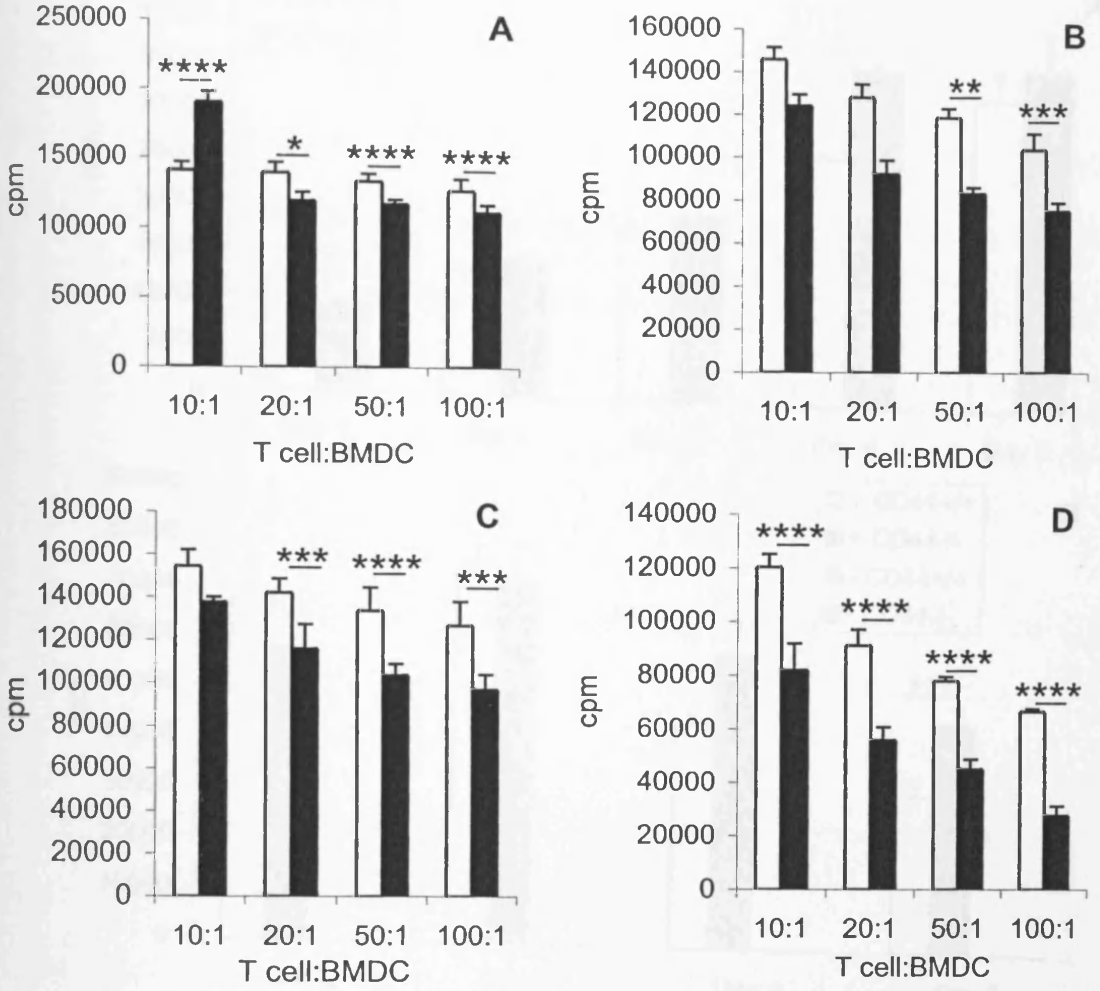

Figure 6.6. Stimulation of $CD8^+$ 2C T cells by $CD44^{+/+}$ and $CD44^{-/-}$ BMDCs.

$CD44^{+/+}$ and $CD44^{-/-}$ BMDC's were incubated with a varying amount of 2C peptide before being added to 2C cells at various ratios. A, Stimulators were incubated with 0.2nM of protein. B, Stimulators were incubated with 0.1nM of peptide. C, Stimulators were incubated with 0.05nM of peptide. D, Stimulators were incubated with 0.01nM of peptide. The data are mean ± s.e.m. from 4 identical wells, using cells pooled from 3 mice. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$. The overall statistical difference between the $CD44^{+/+}$ and $CD44^{-/-}$ cells is $P<0.001$. The black bars are $CD44^{-/-}$ and the white bars are $CD44^{+/+}$.

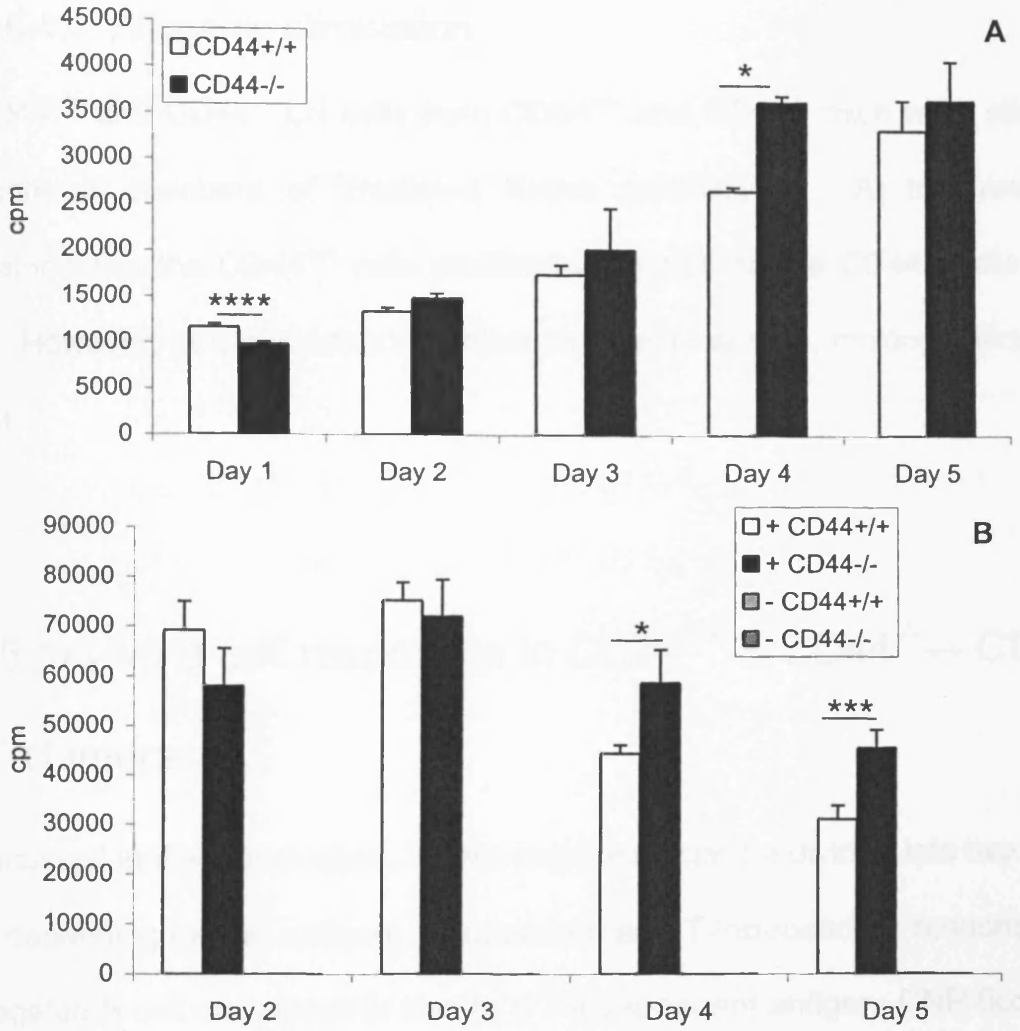

Figure 6.7. Stimulation of $CD44^{+/+}$ and $CD44^{-/-}$ T and B cells from $CD44^{+/+}$ & $CD44^{-/-}\rightarrow CD44^{+/+}$ chimeras.

A, Proliferation over time of $CD44^{+/+}$ or $CD44^{-/-}$ sorted, pooled chimeric spleen cells which were stimulated using anti-IgM immobilised antibody. B, Proliferation over time of $CD44^{+/+}$ or $CD44^{-/-}$ sorted, pooled chimeric LN cells which were stimulated using anti-CD3 & anti-CD28 immobilised antibody (+) or left unstimulated (-). The data are mean ± s.e.m from 4 identical wells, from a group of 3 chimeras. P values, 2-sample t test; *, $P<0.05$, ***, $P<0.005$, ****, $P<0.001$.

6.4.1. Allogenic stimulation

10^5 $CD44^{+/+}$ and $CD44^{-/-}$ LN cells from $CD44^{+/+}$ and $CD44^{-/-}$ mice were stimulated with different numbers of irradiated Balb/c splenocytes. At two responder: stimulator ratios the $CD44^{+/+}$ cells proliferated more than the $CD44^{-/-}$ cells (Figure 6.4C). However, at other ratios no difference was observed, making interpretation difficult.

6.5. *In vivo* B cell responses in $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras

As discussed in the introduction, B cells responses can be divided into two different types depending on the antigen: T-dependent and T-independent responses. We investigated B cell responses *in vivo* to a T-independent antigen: DNP-ficoll, and a T-dependent antigen: chicken gamma globulin (CGG). The B cell response was measured by antibody production, which was aided by the fact that the $CD44^{+/+}$ and $CD44^{-/-}$ mice have allotypically different Igs. $CD44^{+/+}$ mice are IgH^b, whereas $CD44^{-/-}$ mice are IgH^a.

6.5.1. T-independent B cell responses

Mixed chimeras were immunised with DNP-ficoll, and the serum was collected 10 days post immunisation. In addition, various tissues were removed and DNP-ficoll

specific B cell elispots were performed to enumerate B cells secreting DNP-ficoll specific antibodies.

An allotope and DNP specific ELISA was performed to investigate the ability of $CD44^{+/+}$ and $CD44^{-/-}$ B cells to class switch. Endpoint titre were determined using pre-immune sera as the background (Figure 6.8A). No DNP-Ficoll specific antibodies of any isotype other than IgM were detected. IgM^a or $CD44^{-/-}$ B cell antibodies predominated, although the difference was not statistically significant.

The number of B cells producing DNP-ficoll specific antibodies was determined by a DNP-ficoll B cell specific elispot (Figure 6.8B). There were few specific cells isolated and all produced IgM isotype antibodies. There were more $CD44^{-/-}$ than $CD44^{+/+}$ DNP-ficoll specific cells in the spleen and LNs. In contrast, the BM contained similar numbers of $CD44^{+/+}$ and $CD44^{-/-}$ DNP-ficoll specific B cells.

6.5.2. T-dependent B cell responses

Mixed chimeras were immunised with CGG, and the serum was collected from pre-immunised mice, 10 days post-immunisation, 50 days post-immunisation and 10 days post-secondary-immunisation. Upon sacrifice 10 days after secondary immunisation, various tissues were removed and CGG-specific B cell elispots were performed to enumerate B cells secreting CGG specific antibodies.

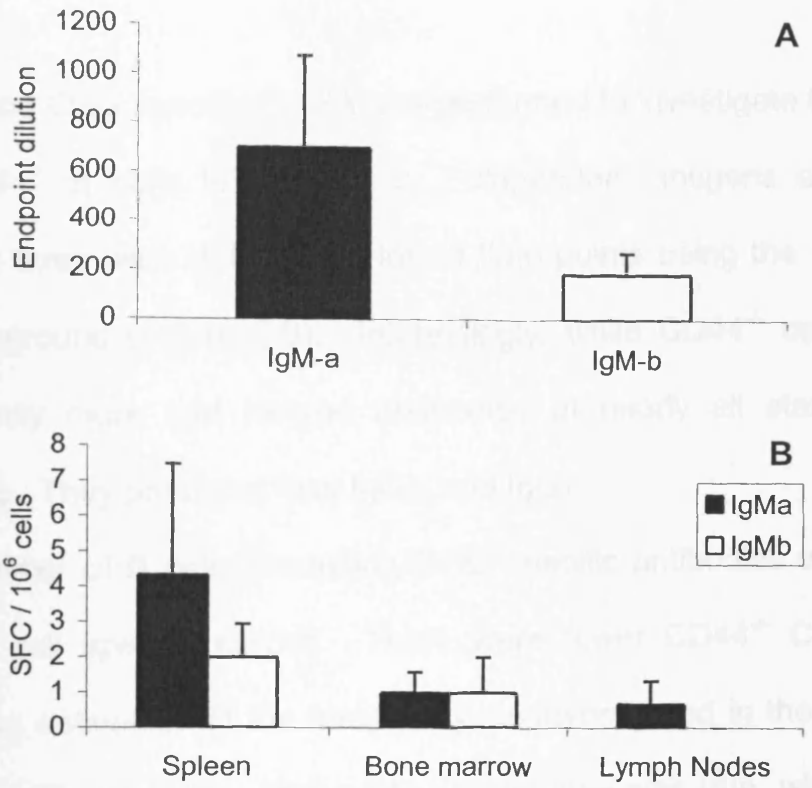

Figure 6.8. $CD44^{+/+}$ and $CD44^{-/-}$ B cell responses to DNP-Ficoll in $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A, Endpoint dilution of $IgM^{a/b}$ DNP-Ficoll specific ELISAs. B, $IgM^{a/b}$ DNP-Ficoll specific spot forming cells from spleen, BM and LN of mixed chimeras. The data are mean ± s.e.m of endpoint dilutions or spot forming cells from a group of 3 chimeras.

An allotope CGG specific ELISA was performed to investigate the ability of $CD44^{+/+}$ and $CD44^{-/-}$ B cells to respond to T-dependent antigens and to class switch. Endpoint titres were determined for all time points using the pre-immune sera as the background (Figure 6.9). Interestingly, while $CD44^{-/-}$ cells mainly produced significantly more IgM isotype antibodies at nearly all stages of the immune response. They produced less IgG_{2a} and IgG_1.

The number of B cells producing CGG specific antibodies was determined by a CGG B cell specific elispot. There were fewer $CD44^{-/-}$ CGG specific B cells producing antibodies of the three isotypes investigated in the spleen of the mixed chimeras (Figure 6.10). In the BM, the reverse was true, with more $CD44^{-/-}$ than $CD44^{+/+}$ CGG specific B cells producing antibodies of all isotypes.

In the draining LN, there was a significantly higher number of $CD44^{-/-}$ cells producing IgM and IgG_1 CGG specific antibodies, but similar numbers of $CD44^{-/-}$ and $CD44^{+/+}$ cells producing IgG_{2a}.

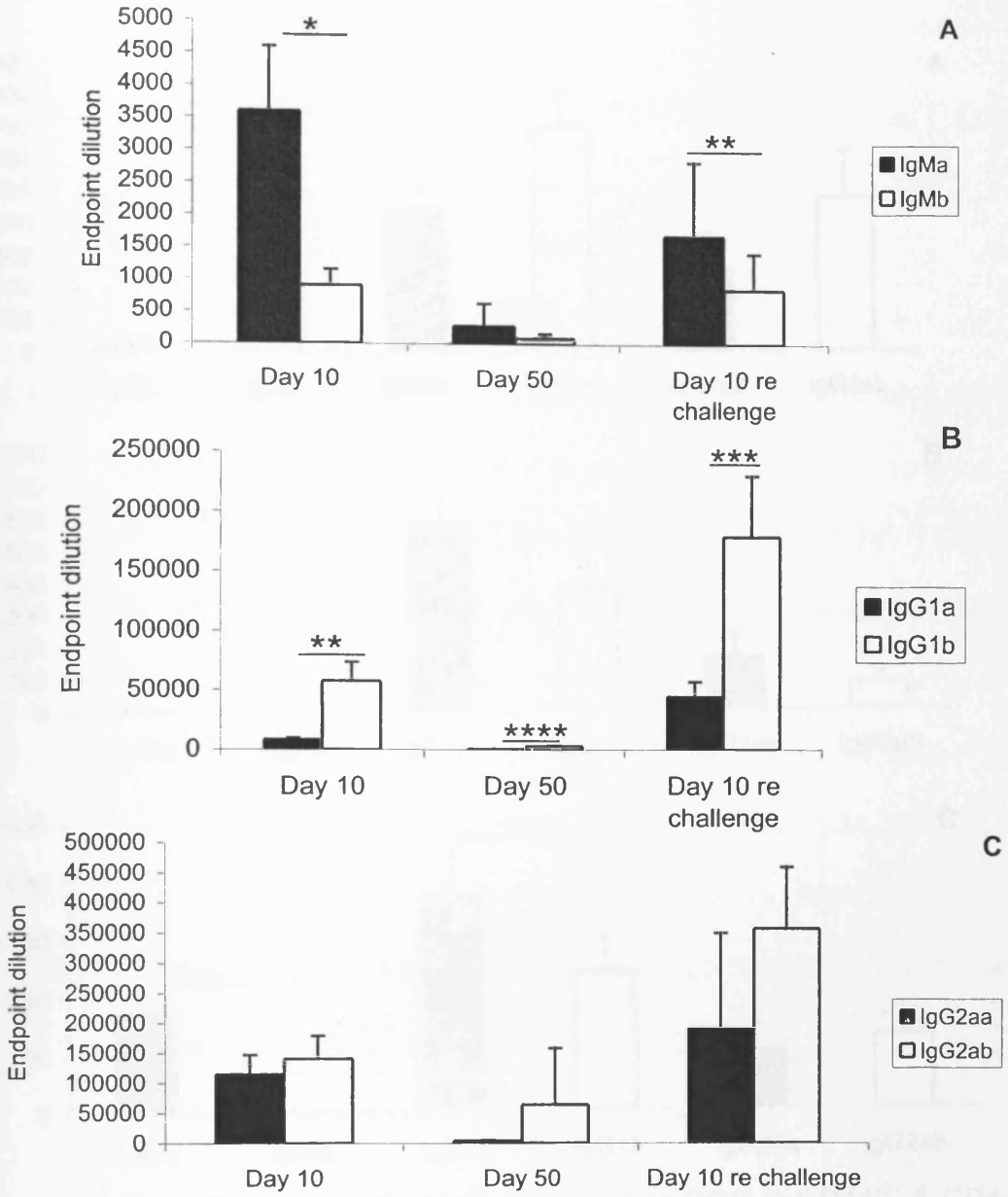

Figure 6.9. $CD44^{+/+}$ and $CD44^{-/-}$ B cell responses to CGG in $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras. I.

A,, Endpoint dilution of CGG specific IgM antibodies from $CD44^{+/+}$ (IgM^{b}) or $CD44^{-/-}$ (IgM^{a}) B cells. B, Endpoint dilution of CGG specific IgG_1 antibodies from $CD44^{+/+}$ (IgG_1^{b}) or $CD44^{-/-}$ (IgG_1^{a}) B cells. C, Endpoint dilution of CGG specific IgG_{2a} antibodies from $CD44^{+/+}$ (IgG_{2a}^{b}) or $CD44^{-/-}$ (IgG_{2a}^{a}) B cells. The data are mean ± s.e.m of endpoint dilutions, from a group of 4 chimeras. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$.

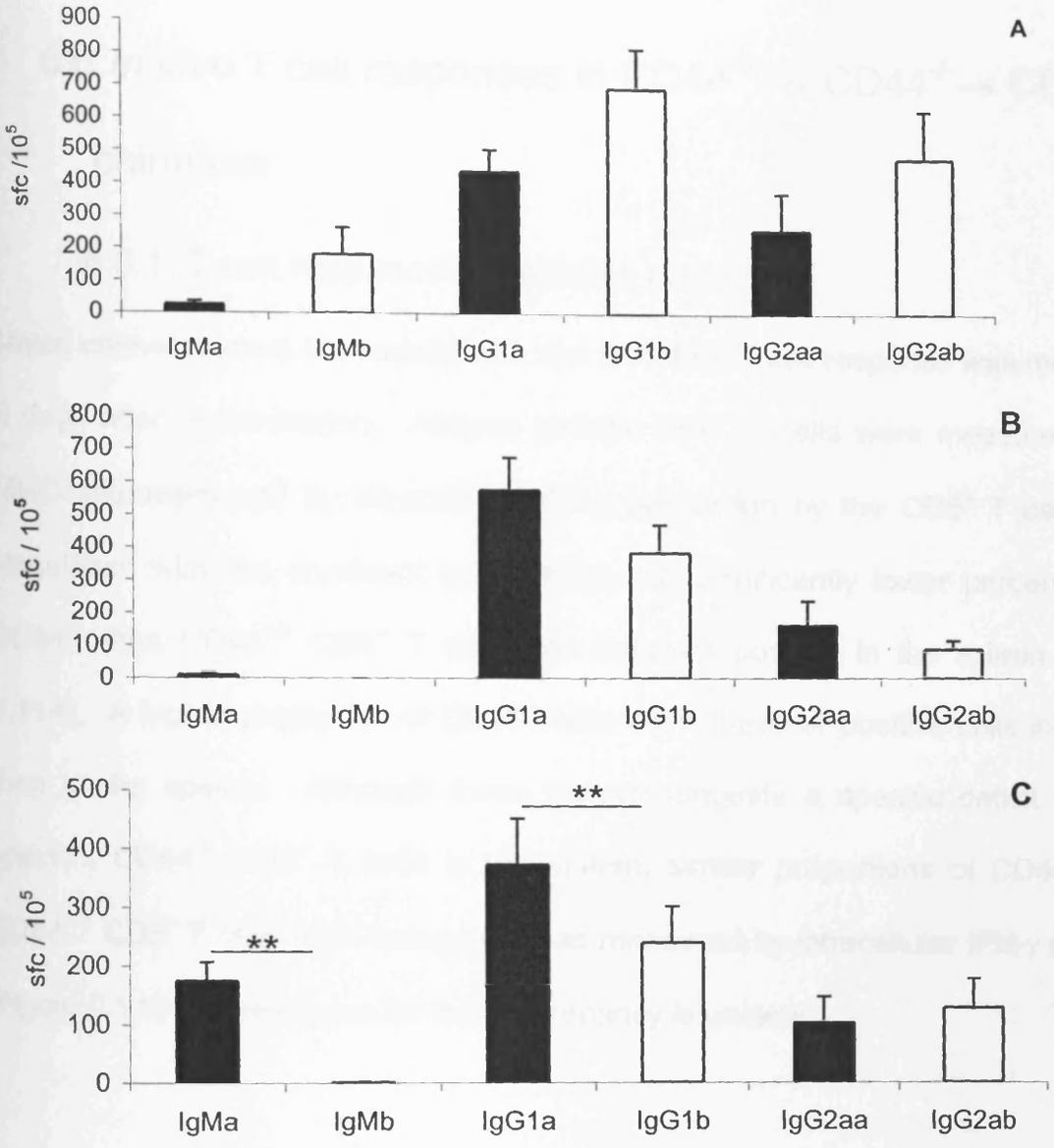

Figure 6.10. $CD44^{+/+}$ and $CD44^{-/-}$ B cell responses to CGG in $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras. II.

A, CGG specific spot forming cells from the spleen of mixed chimeras. B, CGG specific spot forming cells from BM of mixed chimeras. C, CGG specific spot forming cells from the dLN of mixed chimeras. The data are mean ± s.e.m of spot forming cells per 10^5 total cells from a group of 4 chimeras. P values, 2-sample t test; **, $P<0.01$.

6.6. *In vivo* T cell responses in $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras

6.6.1. T cell responses to soluble protein.

Mixed chimeras were immunised with ova and their T cell response was measured 8 days after immunisation. Antigen specific $CD8^+$ T cells were measured using MHCI tetramers and by intracellular IFN-γ production by the $CD8^+$ T cells after stimulation with the dominant ova epitope. A significantly lower percentage of $CD44^{-/-}$ than $CD44^{+/+}$ $CD8^+$ T cells was tetramer positive in the spleen (Figure 6.11A). A higher proportion of $CD44^{-/-}$ cells were tetramer positive cells in the LN than in the spleen. Although these results suggests a specific deficit of ova-specific, $CD44^{-/-}$ $CD8^+$ T cells in the spleen, similar proportions of $CD44^{-/-}$ and $CD44^{+/+}$ $CD8^+$ T cells were ova-specific as measured by intracellular IFN-γ staining (Figure 6.11B). The reason for this discrepancy is unclear.

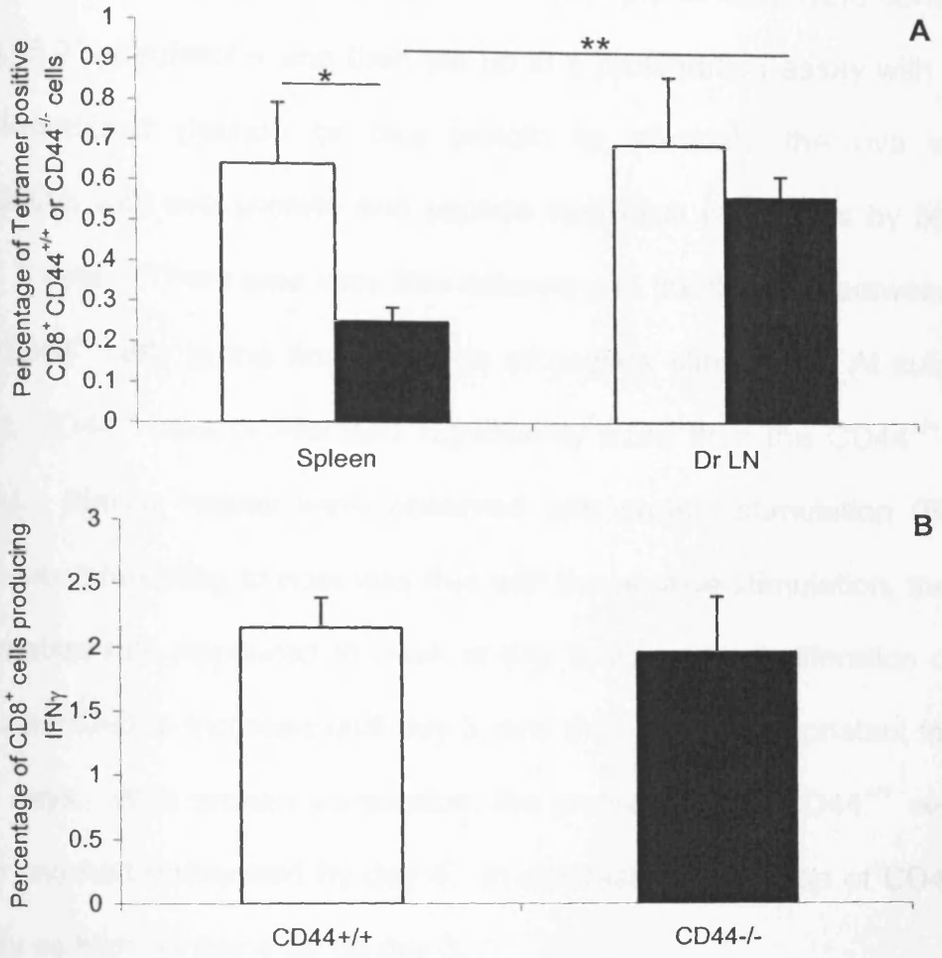

Figure 6.11. $CD44^{+/+}$ and $CD44^{-/-}$ $CD8^+$ T cell responses to ova in $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras. I.

A, Percentage of ova-tetramer$^+$ cells in the $CD8^+$ $CD44^{+/+}$ or $CD44^{-/-}$ populations from the spleen or dLN of mixed chimeras. B, Percentage of IFNγ producing splenic $CD8^+$ $CD44^{+/+}$ or $CD44^{-/-}$ cells in response to ova peptide. The data are mean ± s.e.m from a group of 5 chimeras. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$.

As a further measure of the T cell response, splenic cells were sorted into $Ly5.1^+$ and $Ly5.2^+$ populations and then set up in a proliferation assay with either an ova MHCI-restricted peptide or ova protein to stimulate the ova specific cells, stimulation with ova protein and peptide may elicit responses by both $CD4^+$ and $CD8^+$ T cells. There was very little difference in proliferation between the $CD44^{+/+}$ and $CD44^{-/-}$ cells in the first two days of peptide stimulation. At subsequent time points, $CD44^{-/-}$ cells proliferated significantly more than the $CD44^{+/+}$ cells (Figure 6.12A). Similar results were observed with protein stimulation (Figure 6.12B). What was interesting to note was that with the peptide stimulation, the $CD44^{+/+}$ cell proliferation rate appeared to peak at day 2, whereas proliferation of the $CD44^{-/-}$ cells continued to increase until day 3, and then remained constant for at least two more days. With protein stimulation, the proliferation of $CD44^{+/+}$ cells peaked at day 3 and had decreased by day 4. In contrast, proliferation of $CD44^{-/-}$ cells was equally as high on day 4 as on day 3.

6.6.2. T cell responses to LCMV

Mixed chimeras were infected with lymphocytic choriomeningitis virus (LCMV), and sacrificed 8, 14, 20, and 50 days post-infection. Pieces of spleen were taken and frozen so that the viral titre could be measured. Virus was found in 2 of the 3 chimeras sacrificed at day 8, and in 1 of 2 chimeras at day 14, but was undetectable by day 20, indicating that the mice could successfully clear the virus (Figure 6.13).

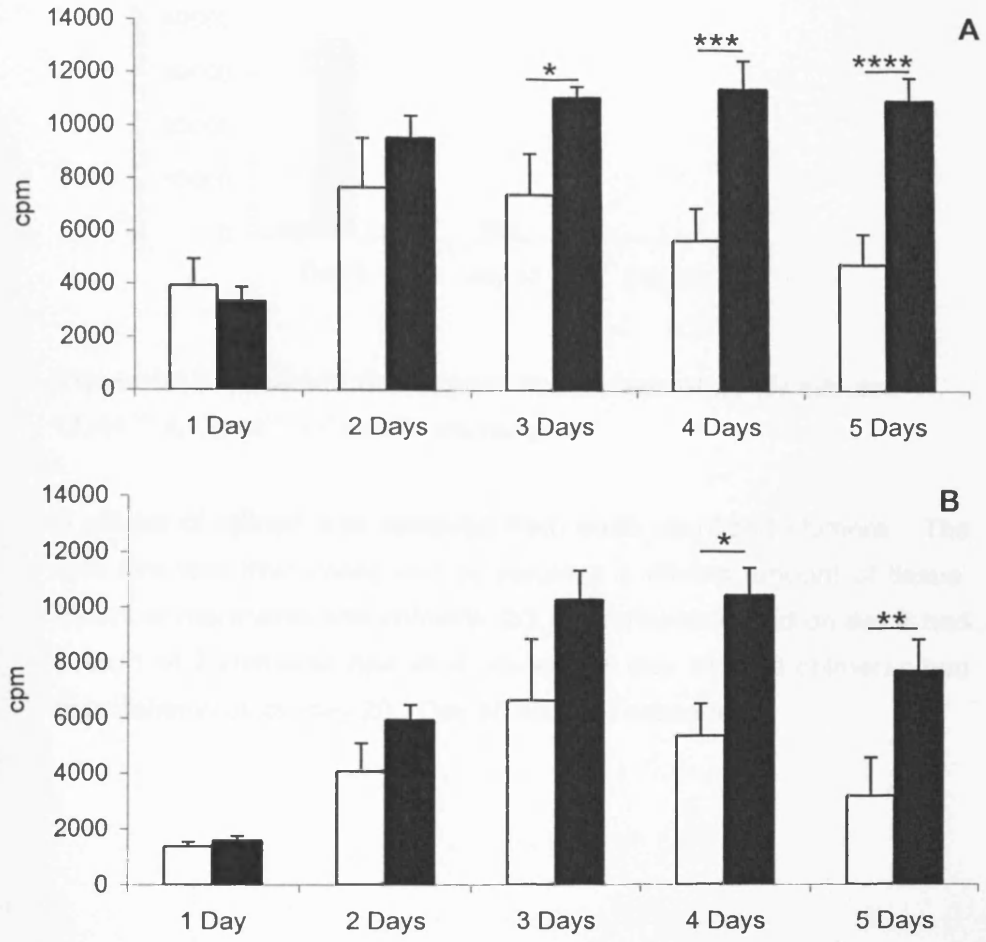

Figure 6.12. $CD44^{+/+}$ and $CD44^{-/-}$ $CD8^{+}$ T cell responses to ova in $CD44^{+/+}$ & $CD44^{-/-}\rightarrow$ $CD44^{+/+}$ chimeras. II.

A, Proliferation of sorted $CD44^{+/+}$ and $CD44^{-/-}$ cells stimulated with an CD8 ova peptide. B, Proliferation of sorted $CD44^{+/+}$ and $CD44^{-/-}$ cells stimulated with a ova protein. The data are mean ± s.e.m from a group of 5 chimeras, each with a triplicate well.. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$, ****, $P<0.001$. The white bars are $CD44^{+/+}$ and the black bars are $CD44^{-/-}$.

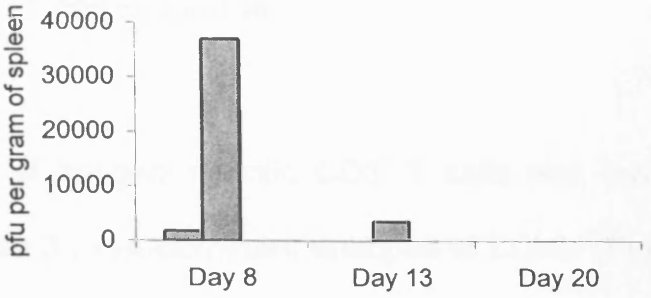

Figure 6.13. Individual viral titration from spleen of LCMV infected $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A pieces of spleen was removed from each sacrificed chimera. The viral titre was then measured by plaquing a diluted amount of tissue. Each bar represents one chimera. 2/3 chimeras sacrificed on day 8 had virus, 1 of 2 chimeras had virus present on day 13. 0/3 chimeras had detectable virus on day 20. Day 50 was not examined.

6.6.2.1. $CD8^+$ T cell response

The frequency of antigen specific $CD8^+$ T cells was investigated using a mix of tetramers for the 3 most-dominant epitopes of LCMV (Figure 6.14). In the spleen there was a significantly higher percentage of tetramer positive $CD8^+$ T cells in the $CD44^{+/+}$ population than in the $CD44^{-/-}$ population (Figure 6.14A). In the LNs a lower percentage of tetramer positive $CD44^{-/-}$ cells was also observed at early (day8 and day 13) but not late times post-infection (Figure 6.14B). In the blood, due to the difficulties in recovering blood from sick animals, it was only possible to perform analysis on 2 chimeras, and therefore the results were not always statistically significant. Nevertheless, there was a higher percentage of tetramer positive $CD44^{+/+}$ than $CD44^{-/-}$ cells at all time points (Figure 6.14C).

To compare the kinetics of the appearance/disappearance of $CD44^{+/+}$ and $CD44^{-/-}$ antigen specific cells, the proportion of tetramer positive cells was plotted against time (using different scales for $CD44^{+/+}$ and $CD44^{-/-}$ cells) (Figure 6.15). Within the spleen, the $CD44^{+/+}$ and $CD44^{-/-}$ cell kinetics appeared to be identical for the first 3 weeks post infection. However, by day 50, the percentage of $tetramer^+$ $CD44^{+/+}$ cells, continued to decrease. Whereas the percentage of $tetramer^+$ $CD44^{-/-}$ cells appeared to increase compared to that at day 20 (Figure 6.15A). These differing trends were also observed in the blood (Figure 6.15C) and to a lesser extent within the LNs (Figure 6.15B).

These results suggest that there may be differences in the homeostatic survival and/or migratory patterns of $CD44^{+/+}$ and $CD44^{-/-}$ memory $CD8^+$ T cells.

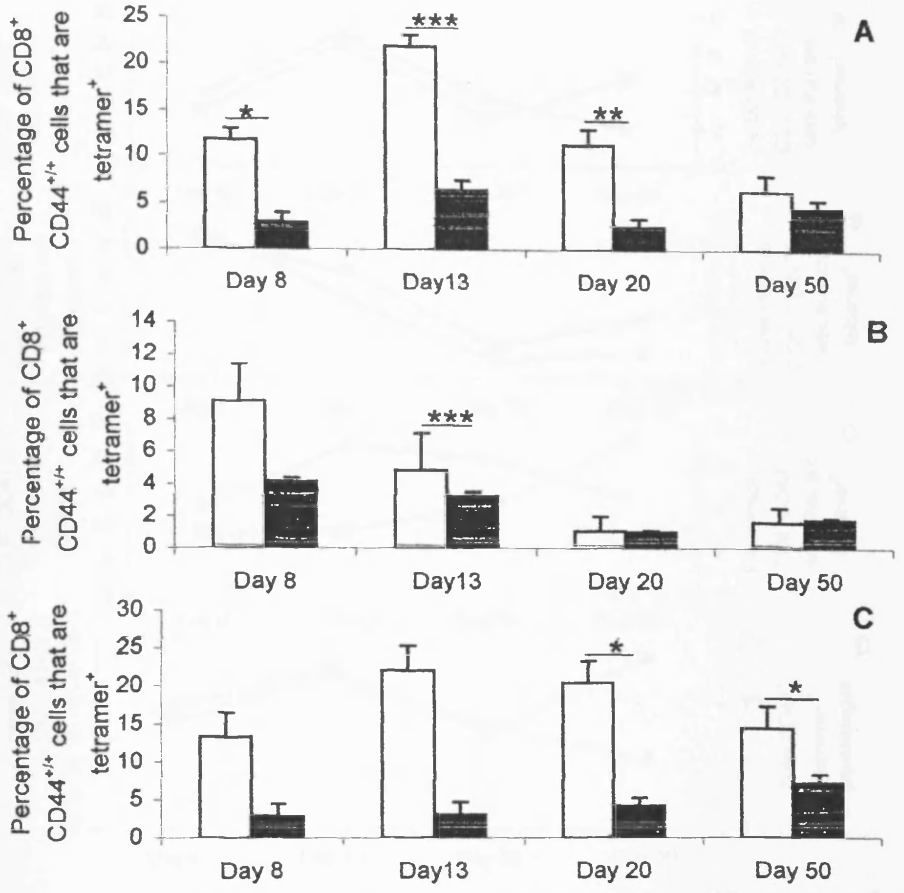

Figure 6.14. $CD8^+$ T cell response to LCMV in $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras. I.

A, Percent of $CD8^+$ $CD44^{+/+}$ or $CD44^{-/-}$ that are tetramer positive in the spleen at various times after infection. B, Percent of $CD8^+$ $CD44^{+/+}$ or $CD44^{-/-}$ that are tetramer positive in the LN at various times after infection. C Percent of $CD8^+$ $CD44^{+/+}$ or $CD44^{-/-}$ that are tetramer positive in the blood at various times after infection. The data are mean ± s.e.m. Day 8 and 13 obtained from 3 chimeras, except blood where from 2. Day 20 obtained from 3 and day 50 obtained from 4 chimeras. P values, 2-sample t test; *, $P<0.05$, **, $P<0.01$, ***, $P<0.005$.

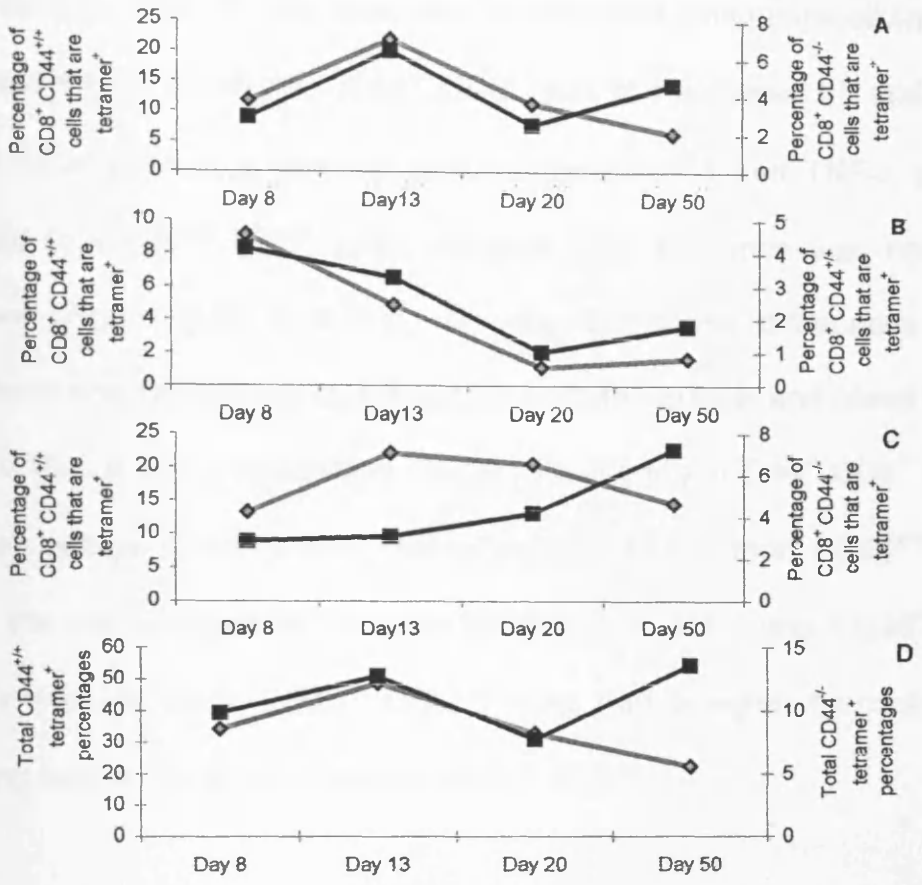

Figure 6.15. $CD8^+$ T cell response to LCMV in $CD44^{+/+}$ & $CD44^{-/-}\rightarrow$ $CD44^{+/+}$ chimeras. II.

A, Percent of $CD8^+$ $CD44^{+/+}$ or $CD44^{-/-}$ that are tetramer positive in the spleen at various times after infection. B, Percent of $CD8^+$ $CD44^{+/+}$ or $CD44^{-/-}$ that are tetramer positive in the LN at various times after infection. C Percent of $CD8^+$ $CD44^{+/+}$ or $CD44^{-/-}$ that are tetramer positive in the blood at various times after infection. D, Culmination of tetramer positive cells, from the different organs examined at various times after development. The data are mean. Day 8 and 13 obtained from 3 chimeras, except blood where from 2. Day 20 obtained from 3 and day 50 obtained from 4 chimeras.

Antigen-specific $CD8^+$ T cells were also enumerated using intracellular staining for TNF-α and IFN-γ. On day 8, $CD44^{-/-}$ $CD8^+$ cells in the spleen, LN and blood of the infected mice showed a reduced percentage of IFN-γ and TNF-α positive cells compared to $CD44^{+/+}$ $CD8^+$ cells, although this difference was not statistically significant (Figure 6.16 & 6.17). On day 20, these differences were more pronounced and statistically significant for both the spleen and blood lymphocytes (Figure 6.18 & 6.19). Interestingly, by day 50, although $CD44^{-/-}$ $CD8^+$ T cells had a lower percentage of IFN-γ and TNF-α producing cells than $CD44^{+/+}$ cells in the spleen, the percentages of cytokine producing $CD44^{-/-}$ and $CD44^{+/+}$ cells was similar in the LN, while $CD44^{-/-}$ $CD8^+$ T cells had a higher percentage of IFN-γ producing cells in the blood (Figure 6.20 & 6.21).

6.6.2.2. $CD4^+$ T cell response

The $CD4^+$ T cell response was measured by intracellular cytokine staining after treatment of cells with MHCII restricted LCMV peptides. On day 8 there was no significant difference between $CD44^{+/+}$ and $CD44^{-/-}$ cells in the percentage of $CD4^+$ cells producing IFN-γ or TNF-α in the spleen, LN or blood (Figures 6.22 & 6.23). On day 20, although the $CD44^{-/-}$ $CD4^+$ T cells appeared to have a reduced percentage of both TNF-α and IFN-γ producing cells, in the spleen, LN and blood, these differences again were not statistically significant. By day 50 of the immune response, however a significantly higher proportion of splenic $CD44^{+/+}$ $CD4^+$ T cells expressed TNF-α (Figure 6.27). In the LN, similar proportions of $CD44^{+/+}$ and $CD44^{-/-}$ cells produced IFN-γ and TNF-α in response to peptide stimulation.

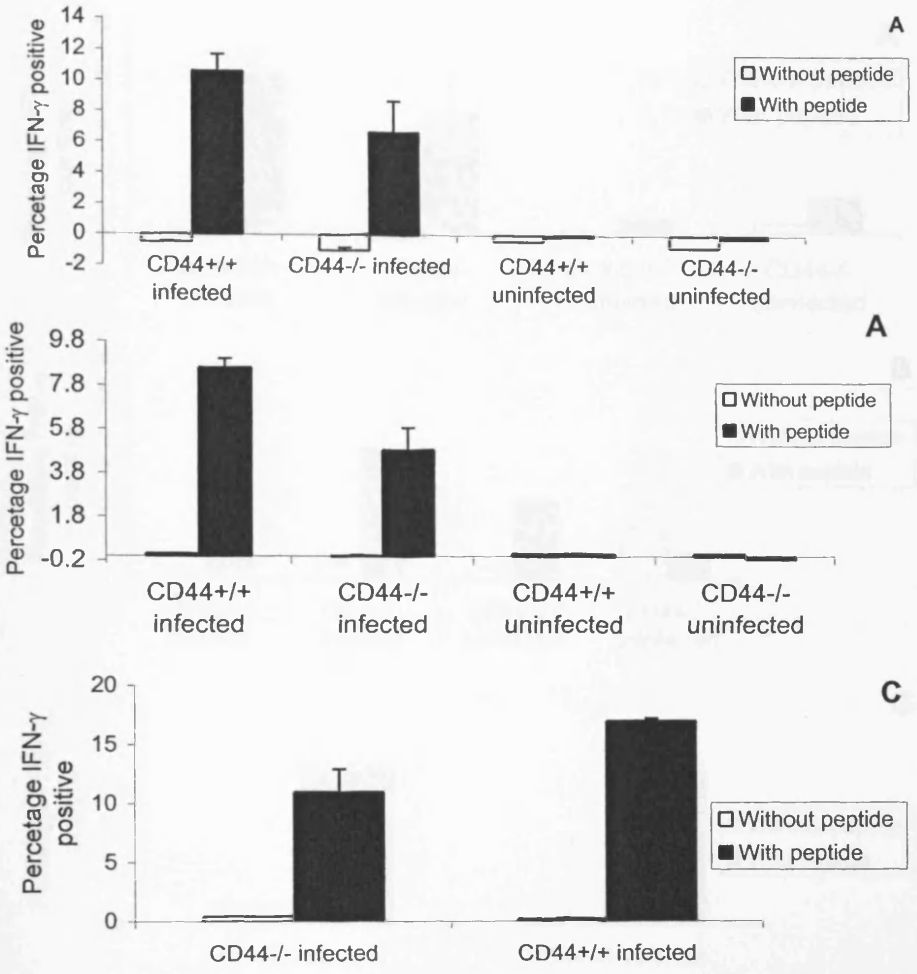

Figure 6.16. $CD8^+$ T cell IFN-γ production on day 8 in response to LCMV in $CD44^{+/+}$ & $CD44^{-/-}$→ $CD44^{+/+}$ chimeras.

A, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from spleen producing intracellular IFN-γ in response to various stimuli. B. Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from LN, producing intracellular IFN-γ in response to various stimuli. C, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from the blood producing intracellular IFN-γ in response to peptide.
The data are mean ± s.e.m. Uninfected 1 chimera, infected spleen and LN 3 chimeras, blood 2 chimeras. P values, 2-sample t test; ***, $P<0.005$.

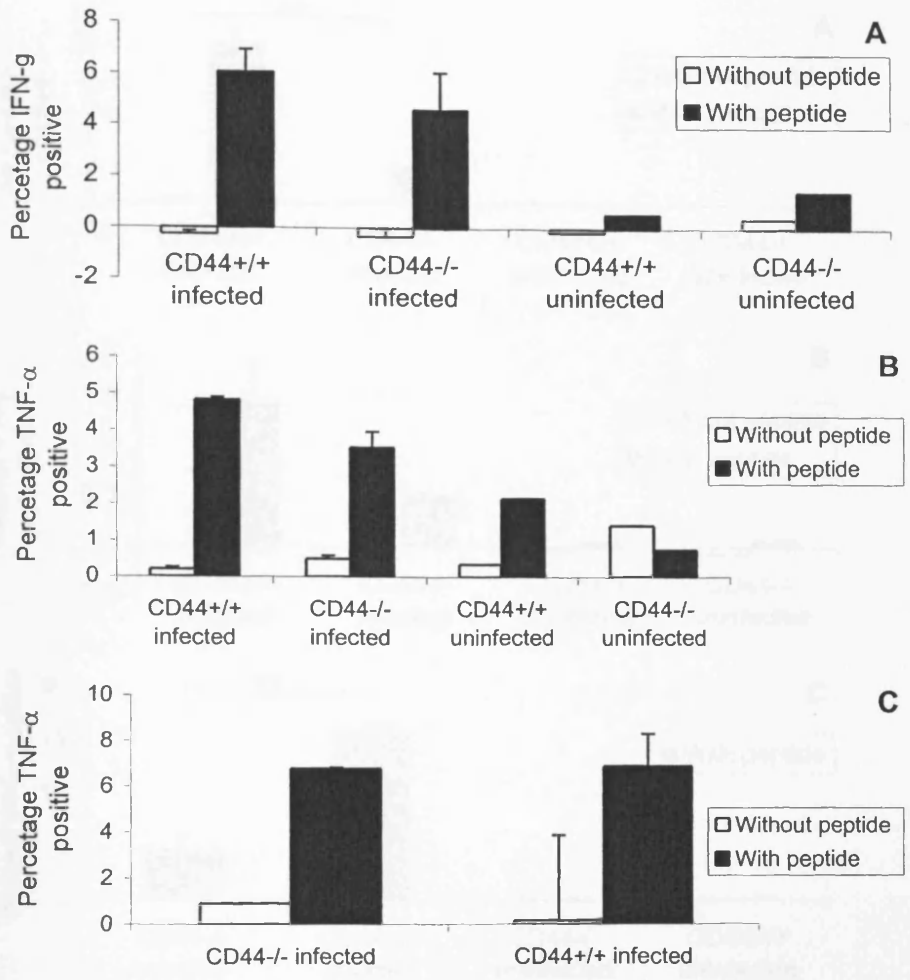

Figure 6.17. $CD8^+$ T cell TNF-α production on day 9 in response to LCMV in $CD44^{+/+}$ & $CD44^{-/-}$→ $CD44^{+/+}$ chimeras.

A, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from spleen producing intracellular TNF-α in response to various stimuli. B. Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from LN, producing intracellular TNF-α in response to various stimuli. C, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from the blood producing intracellular TNF-α in response to various stimuli.

The data are mean ± s.e.m. Uninfected 1 chimera, infected spleen and LN 3 chimeras, blood 2 chimeras. P values, 2-sample t test; ***, $P<0.005$.

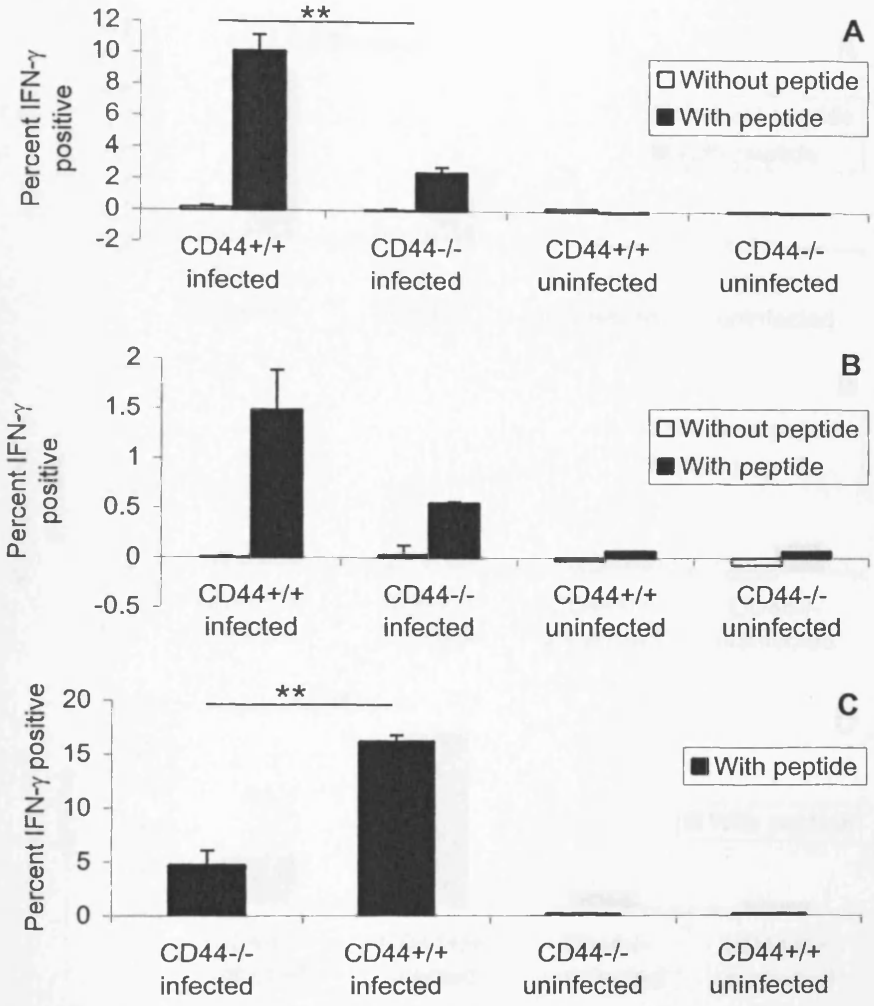

Figure 6.18. $CD8^+$ T cell IFN-γ production on day 20 in response to LCMV in $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from spleen producing intracellular IFN-γ in response to various stimuli. B. Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from LN, producing intracellular IFN-γ in response to various stimuli. C, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from the blood producing intracellular IFN-γ in response to peptide. The data are mean ± s.e.m. Uninfected 1 chimera, infected spleen, LN blood 3 chimeras. P values, 2-sample t test; **, P>0.01 ***, P<0.005.

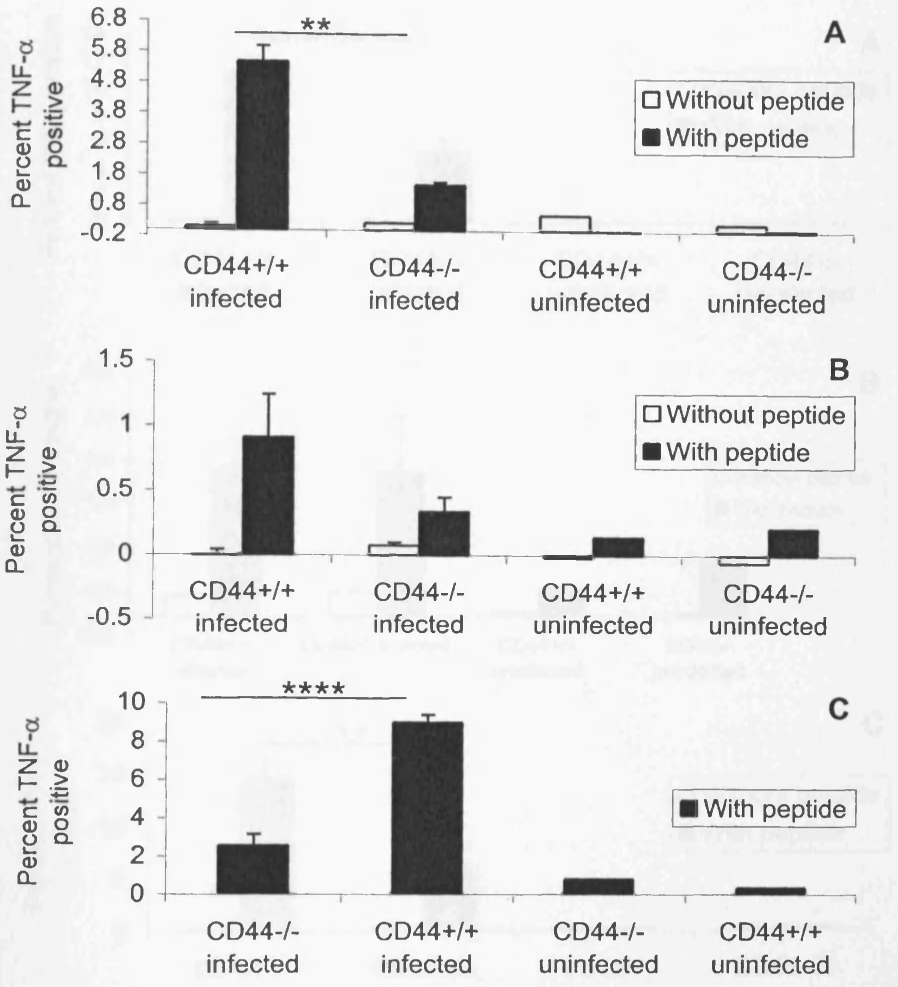

Figure 6.19. $CD8^+$ T cell TNF-α production on day 20 in response to LCMV in $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from spleen producing intracellular TNF-α in response to various stimuli. B. Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from LN, producing intracellular TNF-α in response to various stimuli. C, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from the blood producing intracellular TNF-α in response to peptide. The data are mean ± s.e.m. Uninfected 1 chimera, infected spleen, LN and blood 3 chimeras. P values, 2-sample t test; **, $P<0.01$, ****$P<0.001$.

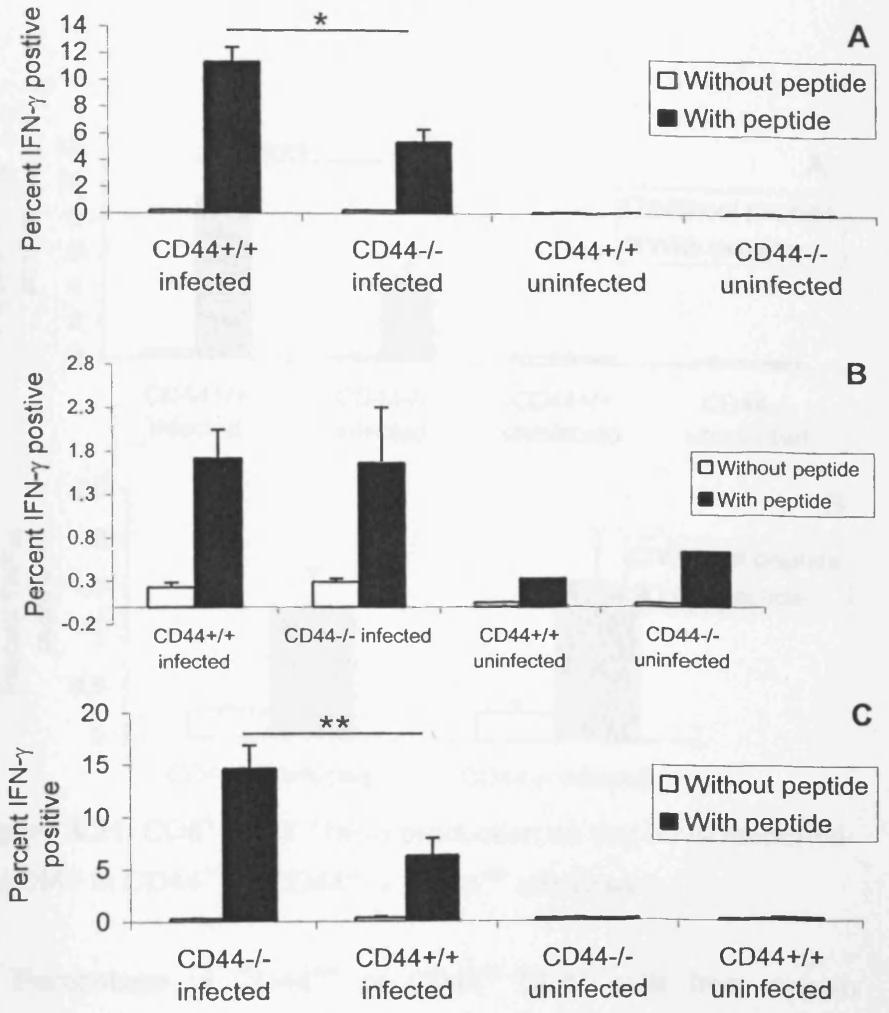

Figure 6.20. $CD8^+$ T cell IFN-γ production on day 50 in response to LCMV in $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from spleen producing intracellular IFN-γ in response to various stimuli. B. Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from LN, producing intracellular IFN-γ in response to various stimuli. C, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from the blood producing intracellular IFN-γ in response to various stimuli.

The data are mean ± s.e.m. Uninfected 1 chimera, infected spleen, LN and blood 4 chimeras. P values, 2-sample t test; *, $P<0.05$.

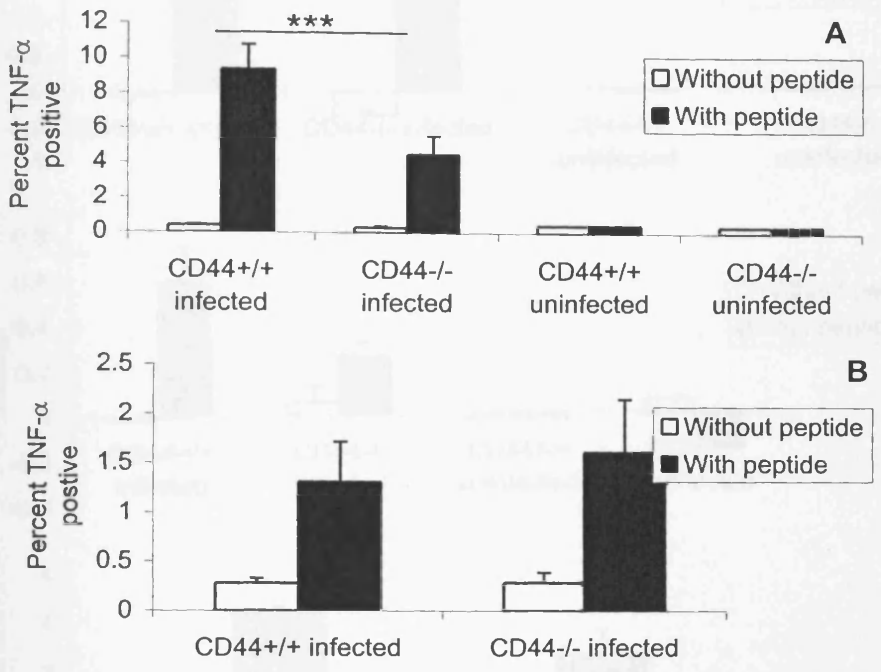

Figure 6.21. $CD8^+$ T cell TNF-α production on day 50 in response to LCMV in $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from spleen producing intracellular TNF-α in response to various stimuli. B. Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD8^+$ cells from LN, producing intracellular TNF-α in response to various stimuli. The data are mean ± s.e.m. Uninfected 1 chimera, infected spleen, LN 4 chimeras. P values, 2-sample t test; ***, $P<0.005$.

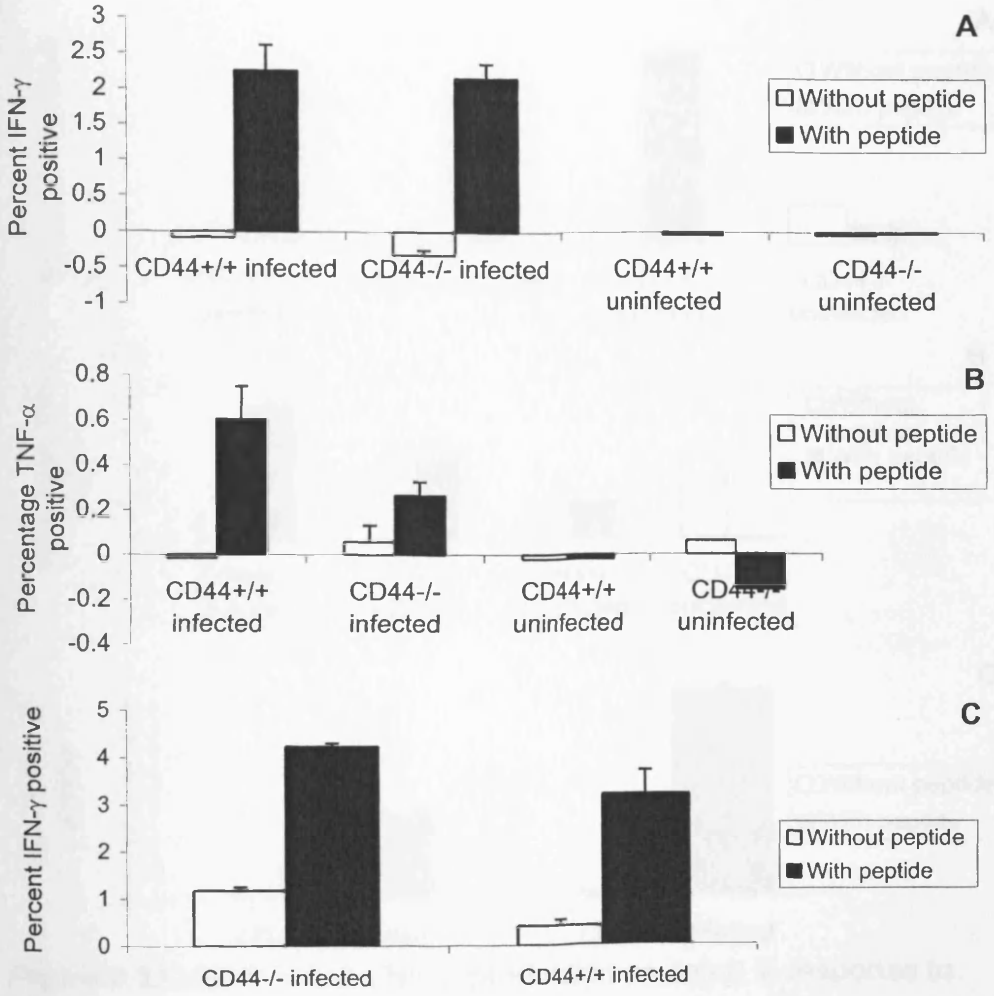

Figure 6.22. $CD4^+$ T cell IFN-γ production on day 8 in response to LCMV in $CD44^{+/+}$ & $CD44^{-/-}$→ $CD44^{+/+}$ chimeras.

A, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from spleen producing intracellular IFN-γ in response to various stimuli. B. Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from LN, producing intracellular IFN-γ in response to various stimuli. C, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from the blood producing intracellular IFN-γ in response to various stimuli.

The data are mean ± s.e.m. Uninfected 1 chimera, infected spleen and LN 3 chimeras, blood 2 chimeras. P values, 2-sample t test; ***, $P<0.005$.

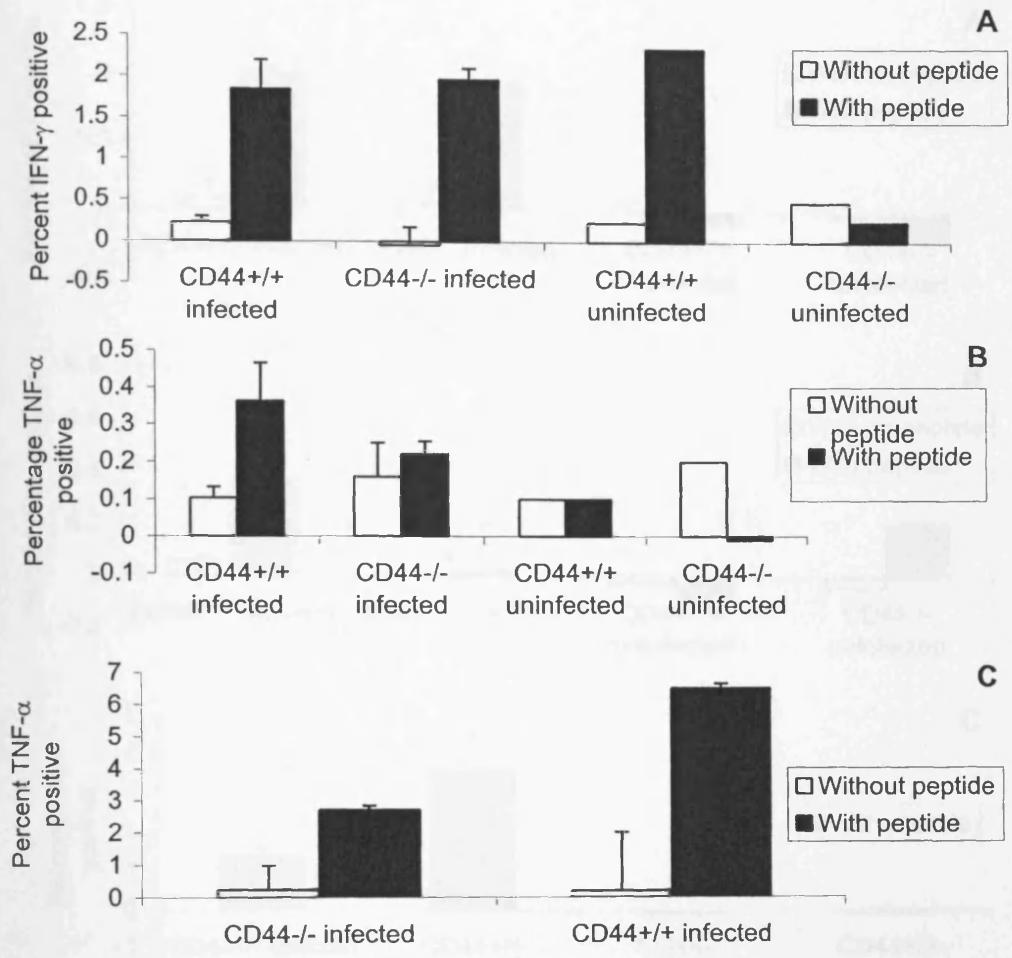

Figure 6.23. $CD4^+$ T cell TNF-α production on day 8 in response to LCMV in $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from spleen producing intracellular TNF-α in response to various stimuli. B. Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from LN, producing intracellular TNF-α in response to various stimuli. C, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from the blood producing intracellular TNF-α in response to various stimuli.

The data are mean ± s.e.m. Uninfected 1 chimera, infected spleen and LN 3 chimeras, blood 2 chimeras. P values, 2-sample t test; ***, $P<0.005$.

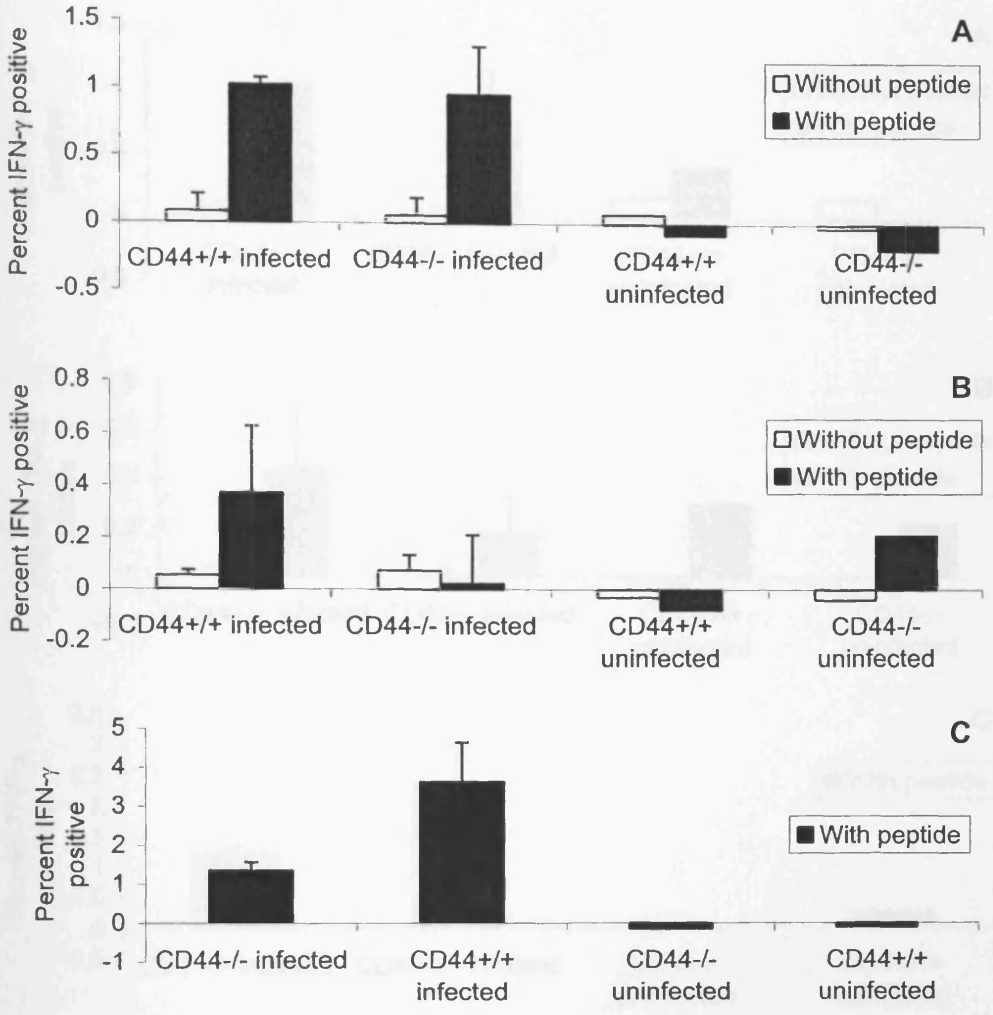

Figure 6.24. $CD4^+$ T cell IFN-γ production on day 20 in response to LCMV in $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from spleen producing intracellular IFN-γ in response to various stimuli. B. Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from LN, producing intracellular IFN-γ in response to various stimuli. C, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from the blood producing intracellular IFN-γ in response to various stimuli.

The data are mean ± s.e.m. Uninfected = 1 chimera, infected spleen and LN = 3 chimeras, blood = 2 chimeras.

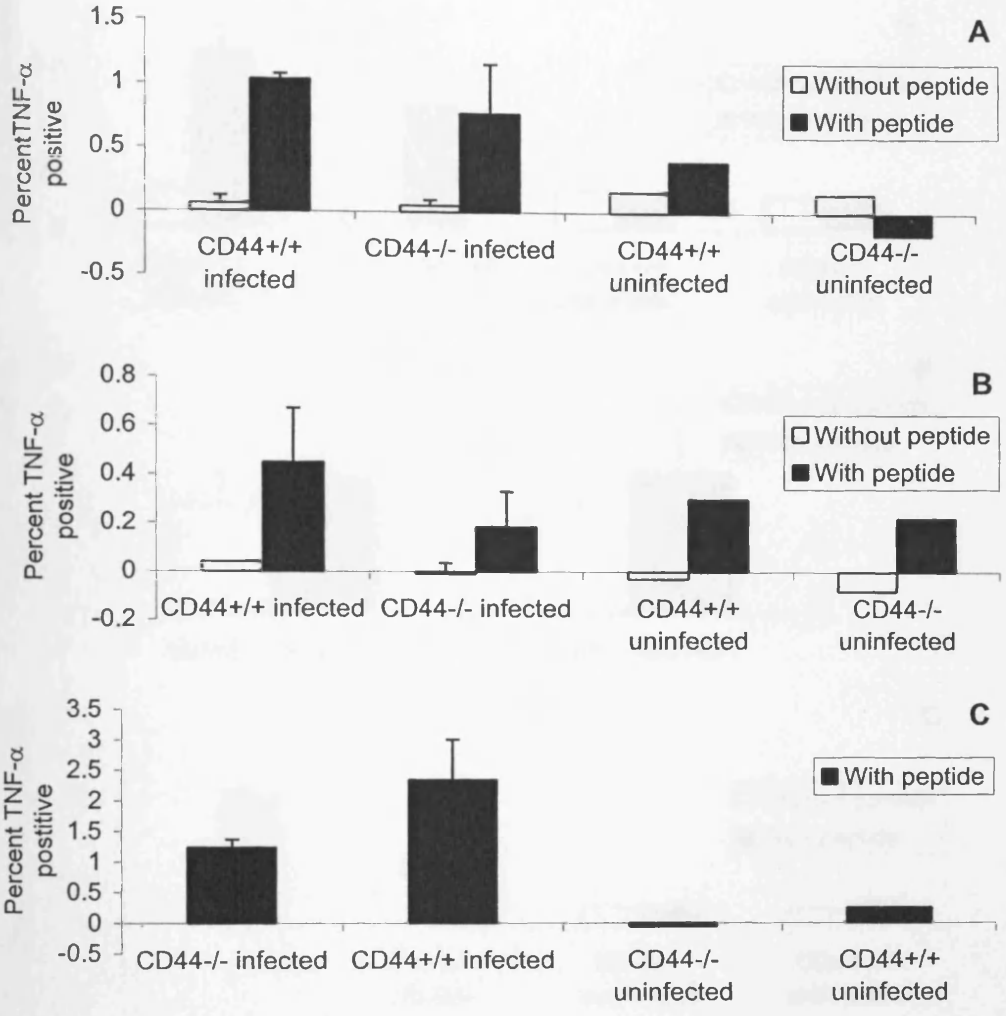

Figure 6.25. $CD4^+$ T cell TNF-α production on day 20 in response to LCMV in $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from spleen producing intracellular TNF-α in response to various stimuli. B. Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from LN, producing intracellular TNF-α in response to various stimuli. C, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from the blood producing intracellular TNF-α in response to various stimuli.

The data are mean ± s.e.m. Uninfected = 1 chimera, infected spleen and LN = 3 chimeras, blood = 2 chimeras.

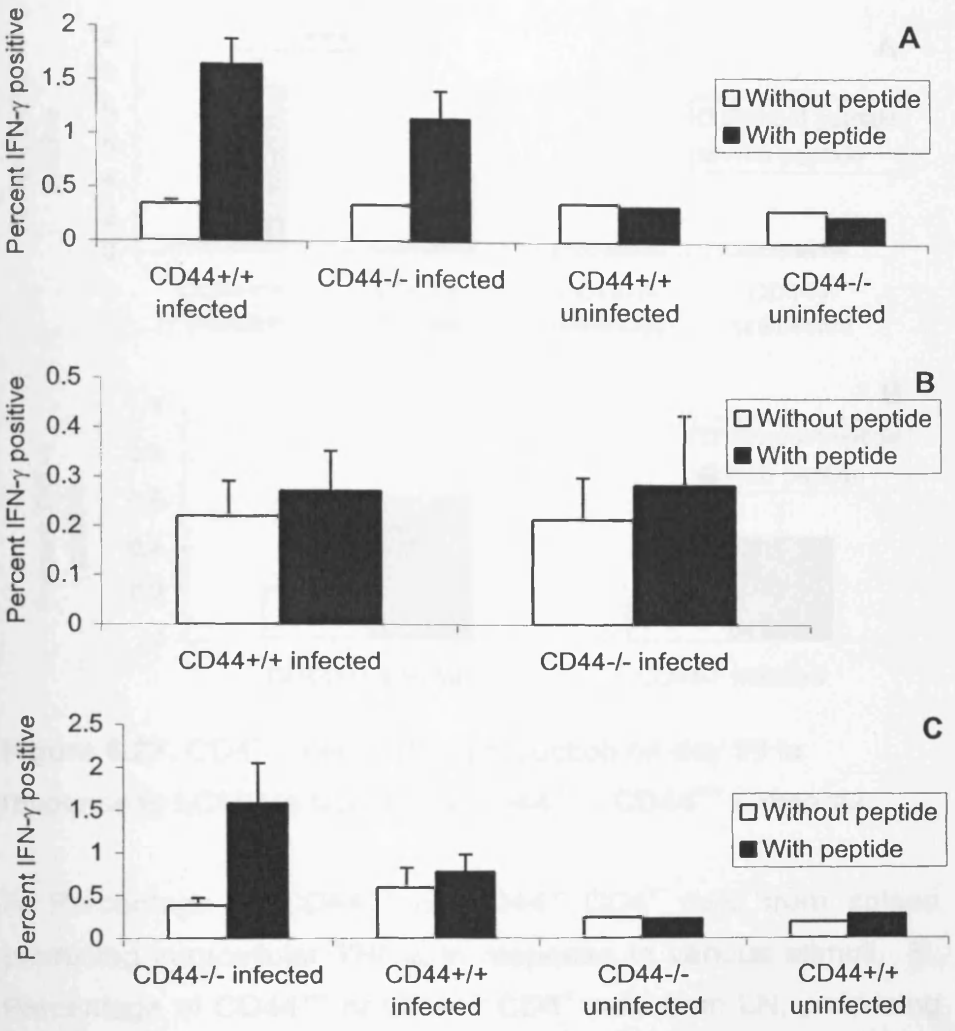

Figure 6.26. $CD4^+$ T cell IFN-γ production on day 50 in response to LCMV in $CD44^{+/+}$ & $CD44^{-/-}$→ $CD44^{+/+}$ chimeras.

A, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from spleen producing intracellular IFN-γ in response to various stimuli. B. Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from LN, producing intracellular IFN-γ in response to various stimuli. C, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from the blood producing intracellular IFN-γ in response to various stimuli.

The data are mean ± s.e.m. Uninfected = 1 chimera, infected spleen and LN = 4 chimeras, blood = 3 chimeras.

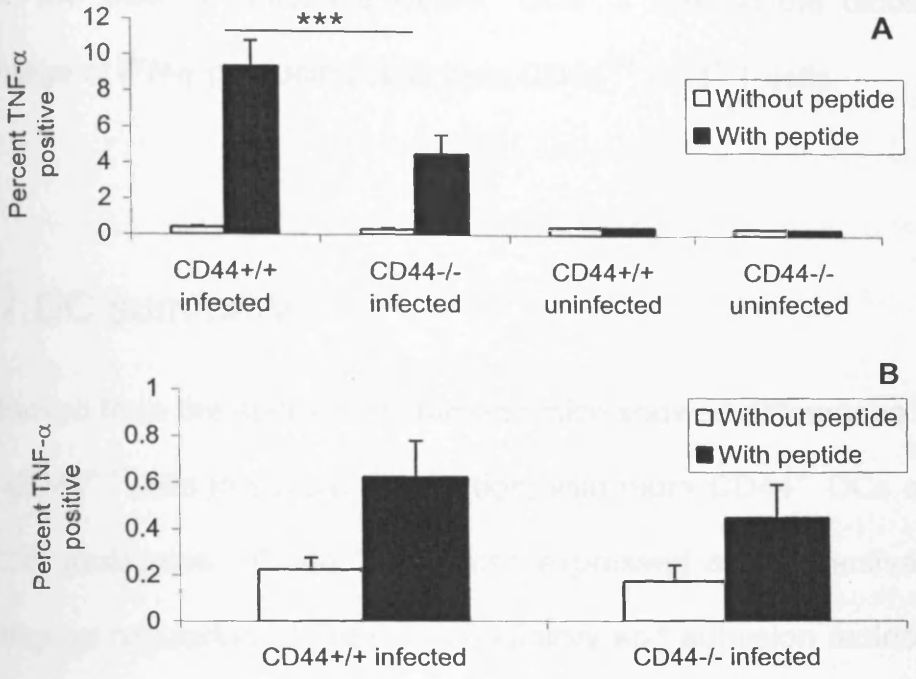

Figure 6.27. $CD4^+$ T cell TNF-α production on day 50 in response to LCMV in $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

A, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from spleen producing intracellular TNF-α in response to various stimuli. B. Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from LN, producing intracellular TNF-α in response to various stimuli. C, Percentage of $CD44^{+/+}$ or $CD44^{-/-}$ $CD4^+$ cells from the blood producing intracellular TNF-α in response to various stimuli.
The data are mean ± s.e.m. Uninfected = 1 chimera, infected spleen and LN = 4 chimeras, blood = 3 chimeras.

As with the $CD8^+$ T cells, the $CD44^{-/-}$ $CD4^+$ T cells in the blood had a higher percentage of IFN-γ producing cells than $CD44^{+/+}$ $CD4^+$ T cells.

6.7.DC summary

DCs derived from the spleens of chimeric mice showed differences between $CD44^{-/-}$ and $CD44^{+/+}$ cells in subset distribution, with more $CD44^{-/-}$ DCs expressing CD4 and CD8 molecules. $CD44^{-/-}$ DCs also expressed a more activated phenotype, exhibiting up regulation of both co-stimulatory and adhesion molecules, especially ICAM-1.

In contrast BMDCs from $CD44^{-/-}$ mice expressed a more immature phenotype than those from $CD44^{+/+}$ mice, having lower expression of both co-stimulatory and adhesion molecules. The exception was ICAM-1, which had a higher expression on $CD44^{-/-}$ BMDCs than $CD44^{+/+}$ BMDCs. As was observed in the splenic DCs.

The ability of BMDCs to take up antigen was briefly examined. The $CD44^{-/-}$ BMDCs could take up more antigen in the time allowed than the $CD44^{+/+}$ cells. As the ability of DCs to take-up antigen decreases with maturity, the greater ability of $CD44^{-/-}$ DCs to take up antigen is consistent with their more immature phenotype.

The ability of $CD44^{+/+}$ or $CD44^{-/-}$ splenic cells and BMDC to stimulate allogenic T cells was examined. $CD44^{-/-}$ splenocytes stimulated a higher level of proliferation by Balb/c responders than the $CD44^{+/+}$ splenocytes. This is consistent with the previous data, which indicated that $CD44^{-/-}$ splenic DCs were phenotypically more mature and therefore expressed more co-stimulatory molecules. Conversely,

$CD44^{+/+}$ BMDCs stimulated the Balb/c responders to proliferate more, in keeping with their more mature phenotype.

The ability of $CD44^{+/+}$ and $CD44^{-/-}$ BMDCs to stimulate antigen-specific $CD4^+$ and $CD8^+$ T cells was investigated using transgenic T cells. There were mixed results. At the high concentrations of ova $CD44^{+/+}$ BMDCs appeared to stimulate more proliferation of OT2 cells, whereas at lower concentrations $CD44^{-/-}$ cells appeared to be more effective stimulators. However, the results obtained using higher concentrations of ova are likely more reliable, since there was a titration effect apparent with changing Tcell: BMDC ratios which was not observed at the lower concentrations.

The ability of $CD44^{+/+}$ and $CD44^{-/-}$ BMDCs to stimulate antigen-specific $CD8^+$ T cells was investigated using 2C transgenic T cells, whose $CD8^+$ T cells are specific for 2C peptide. At nearly all peptide concentrations and T:DC ratios, the $CD44^{+/+}$ cells were better at stimulating the T cells to proliferate.

6.8.B cell summary

Sorted $CD44^{+/+}$ and $CD44^{-/-}$ B cells from mixed chimeras were stimulated *in vitro* by immobilised anti-IgM. As similar experiments had been performed by other groups using $CD44^{+/+}$ and $CD44^{-/-}$ mice, this experiment was only to ensure that B cells obtained from mixed chimeras were capable of responding in a similar fashion as the $CD44^{+/+}$ B cells. This proved accurate and there was very little difference in the proliferation of $CD44^{+/+}$ and $CD44^{-/-}$ B cells in response to immobilised anti-IgM.

The *in vivo* B cell responses demonstrated that $CD44^{-/-}$ cells produced the majority of the antibody in response to the T-independent antigen DNP-Ficoll. The majority of the SPC were found within the spleen, and were most likely B1-B cells or MZB cells. The *in vivo* response to the T-dependent antigen CGG also showed that $CD44^{-/-}$ B cells had a predisposition towards IgM production. Although $CD44^{-/-}$ B cells could class switch to both IgG_1 and IgG_{2a}, there were still significant amounts of IgM present on day 50. Upon re-challenge, the $CD44^{-/-}$ cells appeared to produce primarily IgM rather than IgG_1 or IgG_{2a} like the $CD44^{+/+}$ cells. The localisation of the antibody secreting cells was also different. The $CD44^{+/+}$ IgM secreting cells were mainly found in the spleen after re-immunisation, suggesting that the IgM secreting cells were newly recruited plasma cells. However the majority of $CD44^{-/-}$ IgM secreting cels were found in the BM, implying that these were memory plasma cells. With regards to IgG_1 production there were similar numbers of $CD44^{+/+}$ and $CD44^{-/-}$ IgG_1 secreting B cells found distributed throughout the investigated tissues. This is in stark contrast to the ELISA data which suggests that the $CD44^{-/-}$ B cells produce little IgG_1 antibody in the serum. IgG_{2a}-secreting B cells were also detected throughout the tissues investigated, with $CD44^{+/+}$ and $CD44^{-/-}$ cells having similar numbers. However the ELISA data is inconclusive although it appears as thought the $CD44^{-/-}$ B cells again produce very little IgG_{2a}.

6.9.T cell summary

The ability of the $CD44^{+/+}$ and $CD44^{-/-}$ T cells to respond were tested MLRs using Balb/c splenocytes and T cells from $CD44^{+/+}$ and $CD44^{-/-}$ mice. These results

indicated that as sub-optimum conditions the $CD44^{+/+}$ cells responded better to stimulation than the $CD44^{-/-}$ cells. *In vitro* T cell stimulation of chimeric T cells demonstrated that $CD44^{+/+}$ and $CD44^{-/-}$ cells produced a similar early response but at later points $CD44^{-/-}$ cells proliferated significantly more than $CD44^{+/+}$ cells in response to anti-CD3 and anti-CD28 stimulation.

In vivo T cell responses were investigated in the chimeric mice using ova protein and LCMV. Measurement of the $CD8^+$ T cell response to ova using tetramers indicated that the response was significantly lower for $CD44^{-/-}$ $CD8^+$ T cells in the spleen. There were significantly more $CD44^{-/-}$ ova specific $CD8^+$ T cells present in the LN than in the spleen, possibly reflecting an altered migration of responding cells. However, when the response was investigated using ICS to detect IFN-γ production by the $CD8^+$ cells in the spleen similar percentages of responding $CD44^{-/-}$ and $CD44^{+/+}$ $CD8^+$ T cells were detected. Interestingly, upon re-stimulation *in vitro* $CD44^{-/-}$ T cells were able to maintain proliferation longer than CD44+/+ cells.

The T cell response to LCMV also showed surprising differences between $CD44^{+/+}$ and $CD44^{-/-}$ cells. Again there were fewer $CD44^{-/-}$ antigen specific $CD8^+$ cells present in the spleen, LN and blood on most days after infection. Responses measured by ICS for IFN-γ and TNF-α production mirrored the tetramer data. When the kinetics of the tetramer specific $CD44^{+/+}$ or $CD44^{-/-}$ cells was examined, it appeared as though $CD44^{-/-}$ may persist, at least in the blood longer than CD44+/+ cells. A reduced response among $CD44^{-/-}$ cells was also indicated for $CD4^+$ T cells, where more $CD44^{+/+}$ $CD4^+$ T cells producing TNF-α and IFN-γ were detected.

6.10.Discussion

There appeared to be a difference in the contribution of $CD44^{-/-}$ cells to different DC subsets. It is difficult to conclude whether this has any relevance to the developmental pathways of DC lineage, as DC lineages themselves remain poorly understood. The $CD44^{-/-}$ DC also appeared to be more mature *in vivo* than $CD44^{+/+}$ DCs; a hierarchy which was reversed for DC generated *in vitro* from BM precursors. The reasons for this are unknown. DCs can be activated by numerous triggers, including HA. Therefore, it is possible that in *in vitro*-derived $CD44^{+/+}$ DC's receive a stimulus from HA produced by cells in tissue culture. Greater maturation of $CD44^{-/-}$ DC's in vivo could be related to up-regulation of other adhesion molecules such as ICAM-1 on these cells. This could change the migrational properties of the $CD44^{-/-}$ DC and lead to migration-associated maturation.

During the B cell response to DNP-ficoll, $CD44^{-/-}$ cells produced a stronger response than $CD44^{+/+}$ cells. Conversely, during responses to a T-dependent antigen, B cells did not appear to undergo efficient class switching. These results imply that $CD44^{-/-}$ B cells may not interact efficiently with helper T cells. This could be related to altered migration of activated B cells, or to changes in the way T cells and B cells actually interact.

The $CD44^{-/-}$ T cell responses were also lower than expected, although in response to soluble protein a lower response was detected in the spleen but not in the LN. This could be in part due to differences in migrational properties of the $CD44^{-/-}$ cells between the spleen and LN as demonstrated in a previous chapter. It was

surprising to note that the $CD44^{-/-}$ $CD8^{+}$ T cells produced an equivalent percentage of IFN-γ positive cells compared to the $CD44^{+/+}$ $CD8^{+}$ T cells, considering the reduced percentage of tetramer specific cells. Another surprising result was the proliferation data, in which the $CD44^{-/-}$ cells over time produced a higher and more sustained proliferative response than the $CD44^{+/+}$ cells. This could be explained by the potential resistance of $CD44^{-/-}$ cells to AICD.

After infection with LCMV, the $CD44^{-/-}$ $CD8^{+}$ T cells again did not compete well against $CD44^{+/+}$ T cells. This could be due to competition between the $CD44^{+/+}$ and $CD44^{-/-}$ T cells for interactions with DCs. Over time however the LCMV-specific $CD44^{-/-}$ $CD8^{+}$ T cells appeared to persist better than the $CD44^{+/+}$ cells. This could again be a reflection of a relative resistance of $CD44^{-/-}$ T cells to apoptosis. Alternatively, it could be a result of less migration of $CD44^{-/-}$ cells into peripheral tissues.

Therefore, in summary, $CD44^{-/-}$ cells when generated in competition with $CD44^{+/+}$ cells can still respond to *in vivo* stimuli and mount a response, although this response is muted compared to the $CD44^{+/+}$ response. We suggest that in the early stages of the response when in competition the $CD44^{-/-}$ cells are disadvantaged by weaker cell-to-cell contact which allows the $CD44^{+/+}$ cells to mount a quicker and stronger response. However, after the immune response has decreased, the $CD44^{-/-}$ cells, hampered by their inability to migrate into the inflamed tissues, re-circulate, as demonstrated by a significantly increasing percentage of $CD44^{-/-}$ tetramer positive cells present in the blood. As they are

potentially more resistant to AICD, over time they recover their peak percentages due to turnover and a lack of apoptosis and cell death, and preferentially develop into central memory cells.

Another conclusion to be made is that although $CD44^{-/-}$ cells do not have the characteristic T cell activation and memory marker CD44, this does not prevent them from forming memory T or B cells.

7. General discussion.

7.1. Phenotype

The aim of this thesis was to investigate the developmental and functional roles of CD44 on T and B cells. Considering that CD44 had been implicated in a range of lymphocyte activities, published studies on the $CD44^{-/-}$ mice have revealed surprisingly minor defects. Therefore it was decided to generate $CD44^{+/+}$ and $CD44^{-/-}$ mixed bone marrow chimeras. These mice revealed a deficiency in the production of $CD44^{-/-}$ T cells, and, amongst T cells a bias towards increased generation of $CD4^{+}$ and more especially $CD4^{+}$ $CD25^{+}$ T cells. This was accompanied with an excess of $CD44^{-/-}$ B cells, especially MZB cells, but also a decrease in B-1a B cells. This phenotype was also observed, when mixed chimeras were generated in $CD44^{-/-}$ hosts. Therefore the phenotype was not dependent on CD44 expression in the host, and probably a result of an inherent disadvantage of the $CD44^{-/-}$ cells themselves. Subsequent experiments then established that this phenotype was only apparent when $CD44^{-/-}$ cells were in competition with $CD44^{+/+}$ cells.

When mixed BM chimeras were challenged with various immune stimuli, it was observed that the $CD44^{-/-}$ B cells had a deficiency in class switching and appeared to produce less antibody to T dependent antigens, even though they had similar numbers of antigen specific B cells. The response of $CD44^{-/-}$ $CD8^{+}$ T cells was also diminished compared to $CD44^{+/+}$ cells after challenge with ova or LCMV. In

response to LCMV infection, the $CD44^{-/-}$ T cells had an even greater reduction in IFN-γ and TNF-α producing cells than tetramer binding cells, although this was not observed after ova immunisation.

In vitro studies demonstrated that $CD44^{-/-}$ BMDCs were phenotypically more immature than $CD44^{+/+}$ BMDCs. This contrasted with splenic $CD44^{-/-}$ DCs, in which case $CD44^{-/-}$ DCs were more mature than $CD44^{+/+}$ DCs. The phenotypic immaturity of $CD44^{-/-}$ BMDCs was associated with a reduced ability to stimulate $CD4^+$ or $CD8^+$ T cells.

7.2.CD44 in lymphocyte distribution, migration and homing

When mature T and B cells were examined, it was discovered that there were differences in the distribution of $CD44^{-/-}$ cells between the spleen and LN. More $CD44^{-/-}$ B and T cells were found in the LN than the spleen. $CD44^{-/-}$ and $CD44^{+/+}$ T and B cells also exhibited differences in the expression of adhesion molecules. For B cells, these differences included increased expression of CD62L and $\beta_2$integrin and decreased expression of CD48, LPAM-1, and syndecan. Differences apparent in the $CD44^{-/-}$ T cell population included an increase in CD102, CD11a and $\alpha_4$integrin. These alterations in adhesion molecule expression could help to explain the distribution of the $CD44^{-/-}$ cells. Since CD62L is a LN homing marker, an increase in CD62L on $CD44^{-/-}$ B cells could be why there are more $CD44^{-/-}$ B cells present in the LN.

Upregulation of adhesion molecules could represent a cellular adaptation to the absence of CD44. For example, since CD44 has been demonstrated to interact with CD11a on the surface of cytotoxic cells (177), it is possible that without CD44, there is a compensatory increase in CD11a expression. This could also be true for $\alpha_4$integrin expression. CD44 interacts with the $\alpha_4$integrin in phagocytosis, and therefore a lack of CD44 could necessitate a compensatory increase in $\alpha_4$integrin expression.

7.3.CD44 in B cell development

As CD44 had been extensively implicated in BM homing, it was surprising to find that $CD44^{+/+}$ and $CD44^{-/-}$ HSC enriched BM cells homed equally as well to the BM, although more $CD44^{-/-}$ cells were found in the blood after co-injection with $CD44^{+/+}$ cells. Antibody blocking experiments have demonstrated that CD44 is involved in BM homing and attachment to the stromal cells. However CD44 was not the only molecule that has been implicated in BM homing; $\alpha_4$integrin and $\beta_2$intergrin have also been demonstrated to play a role (268, 269).Since mature B and T cells were shown to have increased expression of $\beta_2$integrin and $\alpha_4$integrin respectively, it is possible that these molecules compensate for the lack of CD44 expression. Moreover, B cells have been demonstrated to develop without stromal cell interactions (270). Therefore a poor ability of $CD44^{-/-}$ progenitor cells to interact with BM stromal cells might not lead to a deficiency in $CD44^{-/-}$ B cells.

HSC have also been demonstrated to bind fibronectin, and a failure of HSC to bind fibronectin, leads to their development into myeloid cells. This could be occurring in the $CD44^{-/-}$ mice where there is an increased number of CFU-GM cells present in the BM. In the chimeras, increased expression of $\alpha_4$integrin, which also can bind fibronectin, could prevent a biased differentiation of $CD44^{-/-}$ cells to the myeloid lineage. In addition, triggering of vCD44 regions has also been demonstrated to release myeloid lineage influencing cytokines (198). Therefore, $CD44^{-/-}$ cells may also be less efficient in producing these cytokines (126, 238, 271).

A possible explanation for the efficient production of B cells from $CD44^{-/-}$ progenitors was that an inability to gain entry to the thymus could result in an increased return of these cells to the BM. Chimeras generated in thymectomised mice suggested that this was not the case, since there was no difference in the percentage of $CD44^{-/-}$ cells present in the B cell population between thymectomised and sham-thymectomised mice.

Another factor influencing B vs. T cell development is notch. It is thought that a strong signal between notch and its ligand, which is present on the stromal cell, is thought to promote T cell differentiation. However, if the lack of CD44 expression leads to weak cell-stroma interactions, this could result in weak notch signalling, and inhibit differentiation into T cells by allowing expression of PAX5 and encouraging B cell differentiation.

Notch2 signalling is also thought to promote differentiation into follicular B cells as opposed B-1 and MZB cells (20, 272, 273). If lack of CD44 this leads to weaker interactions with stromal cells, then it would predicted that there would be less notch2 signalling and therefore more B1 and MZB cells (274). This was only partially observed, for there was an increase in MZB, but not a corresponding increase in B-1 cells. Of note, osteopontin deficient mice have a defect in their B1 cells. Osteopontin is a CD44 ligand, which only binds certain forms of CD44. Hence poor interactions between B cells and osteopontin could reduce the efficiency of B-1 cell differentiation (198).

7.4.CD44 in T cell development

Many groups have demonstrated that CD44 is involved in thymic entry. A deficiency in the ability of mature T cells to enter the adult thymus has been observed in $CD44^{-/-}$ mice. However, $CD44^{-/-}$ mice do generate normal numbers of T cells, indicating that there is not an absolute deficiency in the ability of T cell progenitors to enter the thymus. We observed a reduced ability of $CD44^{-/-}$ progenitors to produce T cells in chimeras only when the $CD44^{-/-}$ cells were in competition with $CD44^{+/+}$ cells. To investigate whether diminished production of $CD44^{-/-}$ T cells in mixed chimeras was strictly due to inefficient thymic entry, T cell development was examined after direct i.t. injection of progenitors. Interpretation of these experiments was complicated by the fact that i.t. injected cells also seemed to seed the BM (presumably due to leakage at the site of injection). At early time points (4 weeks), $CD44^{-/-}$ and $CD44^{+/+}$ cells appeared to produce similar numbers of T cells, suggesting that most of the deficit in T cell production by $CD44^{-}$

$^{-/-}$ cells was due to poor thymic entry. However, by 6 weeks post-injection, fewer $CD44^{-/-}$ T cells were observed. Whether this reflected a true deficit in even i.t. injected $CD44^{-/-}$ cells, or was related to re-seeding of the thymus by injected progenitors that had homed to the BM was unclear.

As with B cell homing, CD44 is thought to co-operate with other molecules, including $\alpha_4$integrin and CD11a, to enter the thymus and interact with stromal cells (128, 221, 222, 250). The thymic epithelium has numerous CD44 ligands present, including the CD44v3 ligand heparin sulphate and GAG (275, 276). Fibronectin is also involved in the movement of T cells through the thymus (277, 278). However this is not thought to handicap the $CD44^{-/-}$ progenitors as a) T cells have been shown to develop without migration within the thymus, and b) $\alpha_4$integrin is thought to control movement within the thymus by binding fibronectin. RHAMM has been shown to co-operate with $\beta_1$integrin to bind fibronectin in the thymus (279). Therefore, other molecules could potentially replace the function of CD44 in thymic entry and migration within the thymus. As mentioned previously, mature $CD44^{-/-}$ T cells had an increased expression of $\alpha_4$integrin, $\beta_1$integrin and CD11a in mixed chimeras.

When T cell development was investigated, it appeared that there was a deficiency in the transition between DN2-DN3 and DN4-DP stages of $CD44^{-/-}$ T cell development. Interestingly, it is at these stages where stromal-T cell interaction are thought to be important (280). Therefore, a lack of stromal cell factors could prevent the progression to the next developmental stage. CD44 is normally down regulated as cells progress from DN2 to DN3, consistent with an important role for

CD44 at the DN2 stage. It is possible that the expression of putative compensatory molecules can not be down regulated as CD44 would normally be, and that this would prevent developmental progression.

Weakened notch signalling could also be involved in changes in T cell development observed for $CD44^{-/-}$ cells. Notch signalling is thought to encourage $CD8^+$ T cell development rather than $CD4^+$ T cell development, by increasing the TCR signal strength. Reduced notch signalling would lead to a biased production of $CD4^+$ T cells rather than $CD8^+$ T cells, as observed in the mixed chimeras. Of note, notch has also been proposed to play a role in DN2-DN3 transition.

Regardless of the role of notch, lack of CD44 could more generally lead to weaker TCR signalling and hence favour $CD4^+$ T cell development. CD44 has also been demonstrated to increase TCR strength by not only increasing the TCR-MHC interactions but also by recruiting more kinases into the TCR signalling cascade.

7.5.CD44 and functional responses

Data derived from $CD44^{-/-}$ mice demonstrated that there were no gross abnormalities in their lymphocyte function, and preliminary data from the chimeric mice agreed with this. However, further examination of B cell responses suggested a deficiency in the ability of $CD44^{-/-}$ cells to produce class-switched antibodies. This could be the result of a reduced ability of $CD44^{-/-}$ B cells to associate with T cells, possibly within germinal centres. Conversely $CD44^{-/-}$ B cells were not deficient in their ability to respond to T-independent antigens. Reduced

T-dependent antibody responses could also be due to a deficit in the BCR signalling cascade, since BCR signalling is dependent upon rac signalling, with which the intracellular region of CD44 is involved. The lack of CD44 in the immunological synapse could lead to a limitation of BCR signalling, which in turn could affect the generation of class switched $CD44^{-/-}$ B cells (281, 282).

A deficit was also observed in the ability of $CD44^{-/-}$ $CD8^{+}$ T cells to respond to viral challenge, as shown by reduced frequencies of cytokine producing antigen-specific cells. Osteopontin deficient mice also have an impaired Type 1 immunity to viruses, as they exhibit decreased IFN-γ production. Therefore some of the deficiency demonstrated by $CD44^{-/-}$ cells could be due to their inability to efficiently bind osteopontin.

The relatively poor $CD44^{-/-}$ T cell response could be related to deficits in cell-cell contact and the immunological synapse. As mentioned above, CD44 is thought to stabilise the immune synapse and recruit more intracellular signalling molecules, facilitating a stronger signal. Therefore, a lack of CD44 could lead to weaker TCR signalling and a weaker response. This may have a particular effect on the production of cytokine expressing cells.

There are conflicting reports on the role of CD44 in regulating T cell apoptosis. Some groups have shown that binding CD44 triggered apoptosis (283), but other have demonstrated that it suppressed apoptosis (260). In our experiments $CD44^{+/+}$ and $CD44^{-/-}$ cells were sorted after ova immunisation and then re-stimulated. At late time points after re-stimulation, the $CD44^{-/-}$ cells were clearly proliferating

more, possibly suggesting a deficiency in AICD for the $CD44^{-/-}$ T cells. One reason that $CD44^{-/-}$ cells could be more resistant to apoptosis is that TGF-β1 is necessary to control apoptosis, and it has been demonstrated that $CD44^{-/-}$ cells produces less TGF-β.

In conclusion, although $CD44^{-/-}$ cells can develop into all investigated lymphocyte subsets and can produce functionally responsive cells there is an inherent deficiency in their fitness when placed in competition with $CD44^{+/+}$ cells. CD44 appears to be involved in maintaining cell-cell contact throughout the lifetime of the lymphocyte, thus facilitating interactions between important ligand pairs such as notch-notch ligand, and contributing to specific signalling through antigen receptors. Other molecules can clearly mediate similar functions and compensate in $CD44^{-/-}$ mice. However the normal function of CD44 can be revealed by observing $CD44^{-/-}$ cells in competition with $CD44^{+/+}$ cells.

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9. Appendix 1: cell numbers

9.1. Cell numbers in various tissues in various chimeras

9.1.1. Cell numbers of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

Tissue	Cell number x 10^6	± s.e.m.
Spleen	77.21	11.51
LN	25.81	3.07
BM	60.89	4.32
Thymus	71.74	7.02
Liver	2.5	0.64
Peritoneal Cavity	5.37	1.86
Peyer's Patches	4.25	0.45

9.1.2. Cell numbers of $CD44^{+/+}$ & $CD44^{-/-} \rightarrow CD44^{-/-}$ chimeras.

Tissue	Cell number x 10^6	± s.e.m.
Spleen	104.53	10.7
LN	27.2	3.28
BM	58.75	3.21
Thymus	28.09	3.84

9.1.3. Cell numbers of $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

Tissue	Cell number x 10^6	± s.e.m.
Spleen	123.21	23.1

Tissue	Cell number x 10^6	± s.e.m.
LN	25.43	4.79
BM	63.5	6.84
Thymus	72.7	11.57
Liver	1.8	0.51
Peritoneal Cavity	1.91	0.08
Peyer's Patches	2.45	0.76

9.1.4. Cell numbers of $CD44^{+/+}$ (Ly5.1) & → $CD44^{+/+}$ (Ly5.2) chimeras.

Tissue	Cell number x 10^6	± s.e.m.
Spleen	106.75	11.63
LN	32.69	7.05
BM	66.82	3.60
Thymus	86.25	12.30
Liver	2.22	0.22
Peritoneal Cavity	8.81	1.32
Peyer's Patches	4.63	1.83

9.1.5. Cell numbers of $CD44^{+/+}$ → $CD44^{+/+}$ chimeras.

Tissue	Cell number x 10^6	± s.e.m.
Spleen	94.25	12.33

Tissue	Cell number x 10^6	± s.e.m.
LN	9.32	1.3
BM	70.25	7.02
Thymus	54.25	1.03

9.1.6. Cell numbers of $CD44^{+/+}$ & $CD44^{-/-}\rightarrow CD44^{+/+}$ (1100 rads) chimeras.

Tissue	Cell number x 10^6	± s.e.m.
Spleen	105.8	10.78
LN	39.20	4.49
BM	64.02	5.30
Thymus	83.22	5.12
Peritoneal Cavity	9.61	1.59
Blood	3.64	0.62
Peyer's Patches	4.1	0.95
Liver	3.8	0.20

9.1.7. Cell numbers of $CD44^{+/+}$ & $CD44^{-/-}\rightarrow RAG^{-/-}$ $CD44^{+/+}$ chimeras.

Tissue	Cell number x 10^6	± s.e.m.
Spleen	56.28	10.47
LN	52.11	7.58

Tissue	Cell number x 10^6	± s.e.m.
BM	62.49	6.75
Thymus	43.75	16.14
Peritoneal cavity	9.92	1.62

9.1.8. Cell numbers of xCD44$^{+/+}$ & yCD44$^{-/-}$→ CD44$^{+/+}$ chimeras.

9.1.8.1. 75% CD44$^{+/+}$ & 25% CD44$^{-/-}$→ CD44$^{+/+}$ chimeras.

Tissue	Cell number x 10^6	± s.e.m.
Spleen	113.81	14.51
LN	41.69	3.93
BM	63.15	5.09
Thymus	105.58	8.32

9.1.8.2. 50% CD44$^{+/+}$ & 50% CD44$^{-/-}$→ CD44$^{+/+}$ chimeras.

Tissue	Cell number x 10^6	± s.e.m.
Spleen	92.52	24.13
LN	39.37	4.12
BM	72.36	3.26
Thymus	90.38	18.33

9.1.8.3. 25% $CD44^{+/+}$ & 75% $CD44^{-/-}$→ $CD44^{+/+}$ chimeras.

Tissue	Cell number x 10^6	± s.e.m.
Spleen	56.13	11.82
LN	13.63	2.25
BM	39.4	7.45
Thymus	46.33	13.32

9.1.9. Cell numbers of HSC $CD44^{+/+}$ & HSC $CD44^{-/-}$→ $RAG^{-/-}$ $CD44^{+/+}$ chimeras.

Tissue	Cell number x 10^6	± s.e.m.
Spleen	91.87	8.66
LN	52.35	11.06
BM	75.07	10.61
Thymus	57.31	10.47
Peritoneal Cavity	7.29	1.54

9.1.10. Cell numbers for LM chimeras

9.1.10.1. Cell numbers for LM $CD44^{+/+}$ & LM $CD44^{+/+}$→ $CD44^{+/+}$ chimeras.

Tissue	Cell number x 10^6	± s.e.m.
Spleen	119.4	23.35
LN	35.94	1.96

Tissue	Cell number x 10^6	± s.e.m.
BM	46.51	9.56
Thymus	68.46	7.95

9.1.10.2. Cell numbers for LM $CD44^{+/+}$ & LM $CD44^{-/-} \rightarrow CD44^{+/+}$ chimeras.

Tissue	Cell number x 10^6	± s.e.m.
Spleen	112.18	15.12
LN	32	3.99
BM	71.47	4.94
Thymus	80.61	9.82